ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

20 twenty years

Rivista italiana di scienza degli alimenti

IJFS

Volume XX
Number 1
2008

CHIRIOTTI EDITORI

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 1/2008 - Torino

LETTER FROM THE EDITOR

Dear Subscribers,

It is with great pleasure and satisfaction that I announce the 20th anniversary of the Italian Journal of Food Science. Over the years, the Journal has evolved to attain its current stable and sound position among the world's chief scientific Food Science journals.

The ISI Impact Factors of IJFS have only varied slightly since 1992, the latest being 0.506. This certainly indicates that, thanks to a careful selection of top quality papers, a small journal can play an important role in a specific international editorial field. This could not have been achieved without the hard work of our Staff and, in particular, of our Co-Editor Sister Mary Traynor, whom I would especially like to thank for her unstinting assistance and great expertise.

To mark this special occasion, Chiriotti Editori has enthusiastically adopted my suggestion to put the Journal on the net! Starting with this first issue of 2008, and in addition to the standard printed version, all of our paid subscribers will be receiving a personal username and password to enable them to access the Journal directly from the net. At the same time, for a small fee, we extend this service to all our foreign readers, who can download the pdf version of IJFS.

The success of any scientific Journal is mainly dependent on the scientists who select it for the publication of their work and ideas and on the reviewers who evaluate the papers. We wish to thank the authors who have given us the opportunity to publish their work and to the many reviewers who have contributed their time and effort to improve the papers with their comments and suggestions.

We are extremely grateful to all of you for having chosen our Journal, thus having enabled us to achieve so much and to view the future with great optimism.

Sincerely yours

Paolo Fantozzi
Editor-in-Chief

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

Property of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

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E-mail: info@chiriottieditori.it - URL: www.chiriottieditori.it

Aim: The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions in food science (chemistry, analysis, microbiology), food technology (engineering, processing) and related areas (nutrition, safety, toxicity, physiology, dietetics, economics, etc.). Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

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Frequency: Quarterly - One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor: 0.506 published in the 2006 Journal of Citation Reports, Institute for Scientific Information

Subscription Rate: 2008: Volume XX

PDF version	€ 40.00
Ordinary	€ 150.00
Supporting	€ 1,000.00

IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing.

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PAPER

KINETIC STUDY OF COPPER(II)- AND IRON(III)- CATALYZED OXIDATION OF L-ASCORBIC ACID IN ASPARTAME-CONTAINING AERATED SOLUTION: PROTECTION OF ASCORBIC ACID

STUDIO DELLA CINETICA DELLA REAZIONE DI OSSIDAZIONE DELL'ACIDO L-ASCORBICO, CATALIZZATA DA RAME(II) - E FERRO (III), IN UNA SOLUZIONE AERATA CONTENENTE ASPARTAME: IL RUOLO PROTETTIVO DELL'ACIDO ASCORBICO

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ABSTRACT

The kinetics of uncatalyzed and metal-catalyzed oxidation of ascorbic acid (AA) in aerated aspartame solutions was studied at 0.1 ionic strength; AA was estimated with CUPRAC spectrophotometry. At fixed pH, the AA oxidation rate

RIASSUNTO

È stata studiata la cinetica di ossidazione dell'acido ascorbico (AA), sia senza catalizzatore che in presenza di catalizzatore metallico, in soluzioni aerate di aspartame allo 0,1 di forza ionica. L'AA è stato determinato con la spettro-

- Key words: ascorbic acid, aspartame, catalyzed oxidation, Cu(II), Fe(III), food stability -

constants decreased with increasing aspartame content for a fixed Cu(II) concentration and increased with increasing Cu(II) or Fe(III) ratio for a fixed aspartame level. The stability of copper-aspartame complex inhibited the catalytic activity of copper in AA oxidation. Aspartame, with or without citrate, protected the AA from catalytic oxidation. The protective level of aspartame should be in excess of Cu(II), and close to the AA concentrations to prevent a prooxidative effect of AA.

fotometria CUPRAC. Ad un determinato pH, le costanti di velocità della reazione di ossidazione dell'AA diminuivano all'aumentare del contenuto in aspartame ad una concentrazione fissa di Cu(II) mentre, ad una fissata concentrazione di aspartame, aumentavano all'aumentare della concentrazione di Cu(II) o di Fe(III). La stabilità del complesso rame-aspartame inibiva l'attività catalitica del rame nella reazione di ossidazione dell'AA. L'aspartame, con o senza aggiunta di citrato, esercitava una protezione dell'AA dall'ossidazione catalitica. Per prevenire un effetto proossidativo dell'AA il livello protettivo dell'aspartame devessere in eccesso rispetto al Cu(II) e vicino al livello della concentrazione dell'AA.

INTRODUCTION

Aspartame (N-L-α-aspartyl-L-phenylalanine 1-methyl ester), a dipeptide synthesized from L-phenylalanine and L-aspartic acid, is a low-calorie sweetener widely used in food, soft drinks and dietary products (FATIBELLO-FILHO *et al.*, 1999). The level of aspartame (APM) in ready-to-consume foods is comparatively low (0.045 - 0.06% in beverages), because its sweetening power is about 200 times that of sucrose (WAFWOYO *et al.*, 1999).

Ascorbic acid (AA) is an important water-soluble antioxidant vitamin in the human diet which participates in many biochemical reactions (MARTELL, 1982). In spite of its simple sugar-like structure, its biochemistry is poorly understood due to a very complicated redox chemistry which makes the molecule an interesting and intriguing reducing agent in inorganic systems. At physiological pH, the monohydrogen ascorbate anion undergoes two separate 1-e transfer steps to form dehydroascorbic acid *via* the ascorbate free radical, and it may be reduced back to AA by various cellular reductants (ZÜMREOĞLU-KARAN, 2006). The controversial physiological redox chemistry of AA is focused on its possible antioxidant and prooxidant roles.

Being a potent water-soluble antioxidant, ascorbic acid prevents LDL oxidation by scavenging an array of reactive oxygen and nitrogen species at low concentrations. It may act as a co-antioxidant for α-tocopherol by converting the α-tocopheroxyl radical to vitamin E, but it may also act as a prooxidant in a concentration–dependent manner (BUETTNER and JURKIEWICZ, 1996) in the presence of catalytically active free iron (HALLIWELL and GUTTERIDGE, 1986).

Copper and iron are important transition metals found in many food and pharmaceutical products (HSIEH and HSIEH, 2000). Ascorbic acid is a natural component of many foods and is often added to foods and beverages as a vitamin supplement and antioxidant. As-

corbic acid is a good indicator of the retention of the nutritional quality of fruit and vegetables because it is highly sensitive to temperature, humidity (ROJAS and GERSCHENSON, 1997) and air.

Several studies have addressed the stability of ascorbic acid in aqueous solution in the presence of another food component, including sucrose or aspartame (LAWRENCE and YUAN, 1996; HSIEH and HARRIS, 1987, 1993; BIRCH and PEPPER, 1983). A protective effect of ascorbic acid in the low calorie sweetener 'stevioside' (13-[2-O-β-D-glucopyranosyl-α-glucopyranosyl)oxy]kaur-16-en-19-oic-acid β-D-glucopyranosyl ester) was observed as a significantly delayed rate of degradation of AA (KROYER, 1999). The effect of aspartame (0.06-0.12%) on the ascorbic acid oxidation in Cu–catalyzed and uncatalyzed solutions has been studied, though with some confusing results, and compared to that of sucrose (10-20%) (HSIEH and HARRIS, 1991). As is apparent from its chemical formula (Fig. 1), by means of its carboxylate and amino groups, aspartame can also act as a ligand for crucial metal ions in biological processes. The cupric-aspartame complexes have been studied (AIHARA *et al.*, 1992; KHOLEIF and ANDEREGG, 1997; ÇAKIR *et al.*, 2003). A kinetic study of the decomposition of aspartame in aqueous solution showed that it slowly degrades into different products including some complexing agents such as L-aspartic acid and L-phenylalanine, as expected from its chemical composition (PRUDEL *et al.*, 1986).

The stability of AA with respect to its oxidative degradation in a number of pharmaceutical products containing natural or artificial sweeteners, including infant liquid formulas (ALMEIDA *et al.*, 1999), "hang-over cure" tablets (www.thermonicolet.com), redoxon double action tablets (www.roche.com) or effervescent vitamin C formulations in which bitter tastes are masked (SOHI *et al.*, 2004), has been a matter of debate. The logarithmic rate constant for dehydroascorbic acid formation from AA degradation (Ln k) at a prespecified percentage relative humidity (% RH) decreases linearly with the reciprocal of the absolute temperature (1/T), and Ln k at a fixed temperature increases linearly with % RH (WATERMAN and ADAMI, 2005). This is closely related to the chemical stability of AA in pharmaceutical dosage forms. Complementary food supplements for children, such as vitamins, may be protected chemically (e.g., by adding citrate or EDTA to chelate the oxidation catalyst Fe(III) or Cu(II)) or physically (e.g., by encapsulation to separate the catalyst from the vitamin). To offset vitamin C losses during food processing and storage, between 20-30% excess vitamin C is commonly added so that the minimum desired concentration of vitamin C is maintained during the expected shelf-life of the product (NETSEL *et al.*, 2003).

Generally the stability of AA in pharmaceutical formulations increases if traces of transition metal ions that catalyze autoxidation are sequestered. Ligands with strong complexing groups are able

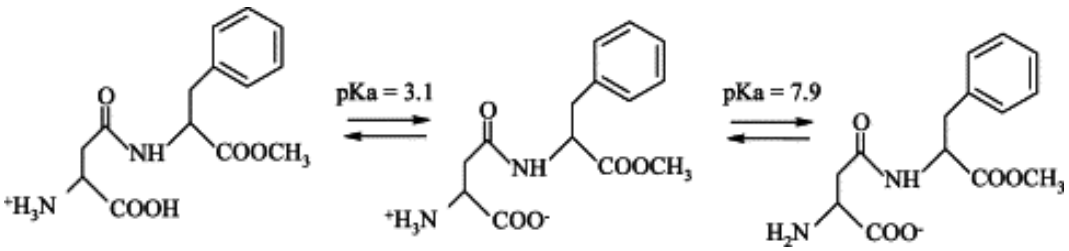


Fig. 1 - Molecular structure and pK_a values of aspartame.

to sequester traces of transition metal ions that are responsible for the catalytic oxidation of AA. Thus, AA substitutes such as sodium- and magnesium- ascorbyl phosphates (Na- or Mg-AP) have been developed by the pharmaceutical industry because they are much more stable hydrophilic ascorbic acid derivatives that can be used in cosmetic products (topical formulations). In such products, the phosphate group near the Na- or Mg-AP cyclic ring protects the enediol system of the ascorbate moiety from oxidation (SPICLIN *et al.*, 2001); in addition the complexing ability of the associated PO_4^{3-} group of these ascorbyl phosphates may act as a sink for traces of transition metal catalysts that would make the compound an antioxidant only when hydrolyzed to AA by enzymes present in the skin.

Traces of transition metal ions like Cu(II) and Fe(III) also act as catalysts in the autoxidation of ascorbic acid. Because of the biochemical importance of AA in the food and pharmaceutical industries, Cu(II)–catalyzed oxidation of AA by molecular oxygen has been extensively studied (KHAN and MARTELL, 1967a,b; OGATA *et al.*, 1968). It has been found that the catalytic efficiency of Cu(II) complexes depends on the nature of the ligands and on the coordination geometry of the metal ion (DAVIES, 1992). The complexation of Cu(II) and Fe(III) by certain ligands such as EDTA, CDTA, NTA, citrate, acetate, tartrate, phthalate, bishistidin and glutathione modifies the catalytic activity (KHAN and MARTELL, 1967a,b; ŞAHBAZ and SOMER, 1993; IMER *et al.*, 2003; SCARPA *et al.* 1996; OHTA *et al.*, 2000). KHAN and MARTELL (1967b) showed that the stability of the cupric or ferric chelate is linearly correlated to its catalytic activity in AA oxidation. However, the findings of HSIEH and HARRIS (1991) on the role of aspartame in accelerating the Cu(II)-catalyzed ascorbic acid oxidation at 30°C were contradictory to this rule. Due to its chelating properties, aspartame should reduce the catalytic activity of copper, and hence lower the oxidation rate of AA. It is, therefore, important to study the Cu(II)– or Fe(III)– catalyzed AA oxidation system in the presence of aspartame. In addition, it is important to stabilize a system composed of cupric or ferric ions and ascorbate with respect to the generation of reactive species, because AA is known to serve as a source of reducing equivalents for the redox cycling of transition metal ions, and this activity has been shown to induce lipid peroxidation in some oxidizing systems (TRIBBLE *et al.*, 1995).

The levels of ascorbic acid in pharmaceuticals, soft drinks and fruit juices can be determined by UV-visible spectrophotometric methods (LAU *et al.*, 1986, 1987; HAREL, 1994). However, the catalytic oxidation of AA in the presence of APM cannot be followed by UV spectrometry because the APM band interferes in the UV region. The objective of this study was to determine the effects of aspartame on the kinetics of uncatalyzed and either Cu(II)- or Fe(III)-ion catalyzed oxidation of AA in aerated and acetate-buffered solutions at 25°C and ionic strength: I=0.1 (KNO_3) using Cu(II)-Nc as the reagent for a spectrophotometric AA assay (GÜÇLÜ *et al.*, 2005). Besides quantifying the AA, the Cu(II)-Nc reagent was modified to assay a wide variety of antioxidants (including flavonoids, vitamin E, β-carotene, uric acid and bilirubin); this is the basis of the CUPRAC method used to measure total antioxidant capacity (APAK *et al.*, 2004, 2005).

MATERIALS AND METHODS

All chemicals were of analytical reagent grade and were supplied by E. Merck (AG, Darmstadt, Germany). L-ascorbic acid (referred to as ascorbic acid in the text) was used without purification. Redoxon chewable tablets (Roche,

Istanbul, Turkey), C-plan chewable tablets (Polifarma, Istanbul, Turkey), and redoxon effervescent tablets (Roche, Istanbul, Turkey) were purchased from local pharmacies. The Redoxon chewable tablets contained 500 mg AA per 1390 mg tablet; the other ingredients were sorbitol, APM, passion fruit aroma and peach aroma. The C-plan chewable tablets contained 500 mg AA per 660 mg tablet; the other ingredients were starch, talc, Mg-stearate, saccarin sodium and peach aroma. The Redoxon effervescent tablets contained 1000 mg AA per 4700 mg tablet; the other ingredients were sorbitol, APM, orange aroma, lemon aroma, and presumably $NaHCO_3$ and citric acid for self-dissolution of the tablet in water by CO_2 evolution. The tablet solutions were prepared in distilled water, double-filtered through a Whatman quantitative filter paper, pH-adjusted, and diluted to the same AA concentration range as the standard solutions of ascorbic acid for kinetic study and comparison. Cu(II)-Nc reagent was used for the AA assay as recommended (GÜÇLÜ *et al.*, 2005). Acetate buffers (10 mM) were prepared according to HSIEH and HARRIS (1993). Deionized distilled water was used to prepare and dilute all solutions.

The absorbances were measured and the spectra taken with an Agilent 8453 UV-Vis spectrophotometer using a pair of matched quartz cuvettes of 1 cm thickness. The pH measurements were made using a 632 Digital pH-meter (Metrohm, Herisau, Switzerland) with a combined glass electrode. The ionic strength of the reaction medium was maintained at I= 0.1 (KNO_3) in order to keep the activity coefficients of the relevant species constant during the kinetic process. All the experiments were carried out at 25°C by means of a thermostatted system (±0.5°C) which contained an immersion circulator (Thermomix 1419 B. Braun model, AG Melsungen, Germany).

Sample preparation and kinetic measurements

All the stock solutions were prepared daily; 7.5 mM Nc solution in ethanol, 10 mM $CuCl_2$ aqueous solution and 1 M ammonium acetate aqueous solution were used in the determination of AA with Cu(II)- Nc reagent.

A stock of either cupric or ferric nitrate solution with a Cu^{2+} or Fe^{3+} concentration of 15.7 μM, APM solution of 10 mM, AA solution of 5.67 mM and an acetate buffer solution of 10 mM were prepared. A sample solution containing 0.1 M KNO_3, 1.5 mM acetate buffer, APM in the concentration range from 0.1 to 4 mM, either Cu^{2+} or Fe^{3+} in the concentration range from 0.157 to 0.628 μM was prepared in a 100 mL volumetric flask, 0.567 mM AA solution was added immediately to the sample solution and the reaction was timed. Sample solution was placed in a 250 mL Erlenmeyer flask in contact with a water bath thermostatted at 25°C. During this time, a stream of air (flow rate = 60 L h^{-1}) was passed through the Erlenmeyer flask and the solution was saturated with oxygen. The air was presaturated with water vapor passed through a wash-bottle. Since the reaction rate is slow compared to the dissolution rate of oxygen, the reacting solution was considered to be saturated with oxygen at all times. After the addition of ascorbic acid, an aliquot of 0.6 mL was taken periodically for spectrophotometric measurement and the pH of the sample solution was recorded. The rate of oxidation was measured by quantifying the residual AA during the course of oxidation. All tests were carried out in triplicate, and the results are presented as the means of the three tests.

Procedure for AA determination

The method used to determine the ascorbic acid concentration is based on the oxidation of AA to dehydroascorbic acid

with a Cu (II) - Nc reagent in an ammonium acetate-containing medium at pH 7, where the maximum absorption wavelength of the formed bis(neocuproine) copper (I) chelate is 450 nm (GÜÇLÜ *et al.*, 2005).

One mL of $CuCl_2$ solution was placed in a test tube, and then 1 mL of Nc, and 1 mL ammonium acetate to bring the final pH to 7; 1.4 mL of water, and 0.6 mL AA solutions were then added by mixing in this order. After two min, the absorbance at 450 nm was recorded against a reagent blank. All solutions in which transition metal-catalyzed autoxidation of AA in the absence or presence of APM were observed were then analyzed for ascorbic acid content.

UV spectroscopic investigation of Cu(II)-aspartame complex stoichiometry

The UV absorbances of 1 mM equimolar mixtures of Cu(II)-nitrate and APM aqueous solutions were recorded in solutions containing 0.1 M KNO_3 at the absorption maximum wavelength of 258 nm against the blank which did not contain Cu(II). (The absorbances of metal-ligand mixtures and of pure APM solutions containing the same amount of ligand with the mixtures were both measured against distilled water, and the difference between the corresponding A_{258} values were recorded *vs* mole fraction of Cu(II)). The stoichiometries of the Cu(II)-APM complexes were studied by Job's method of continuous variations at three discrete pH values 3.2, 4.5 and 5.6 (achieved with acetate-acetic acid buffer) such that 0-10, 1-9, ..., 9-1, and 10-0 mL-mL mixtures of Cu(II)-APM were measured.

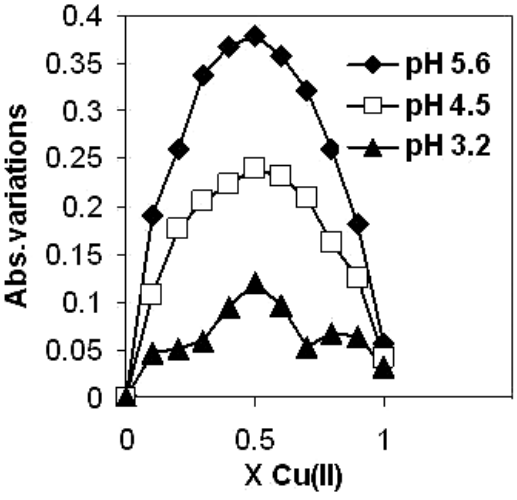


Fig. 2 - Job's curves of continuous variations determined at three different pH values (3.2, 4.5 and 5.6); absorbance at 258 nm recorded *vs* $X_{Cu(II)}$: mole fraction of Cu(II).

RESULTS AND DISCUSSION

Complex stoichiometry

Job's curves for three different pH values are depicted in Fig. 2; in all cases the Cu-APM stoichiometry was found to be 1:1 (mole/mole). This finding is supported by KHOLEIF and ANDEREGG (1997) who confirmed the predominance of the 1:1 binary complex among other species at slightly acidic pH. LAWRENCE and YUAN (1996) formulated a similar 1:1 complex of APM-Cu(II)-OH as an intermediate in AA catalytic oxidation. Since APM is a bidentate chelating ligand, the coordinative unsaturation of Cu(II) in the complex should allow the formation of a ternary APM-Cu-AA transition species that could be responsible for catalytic oxidation in similar systems (KHAN and MARTELL, 1967b; IMER *et al.*, 2003).

Kinetic studies

Theoretical considerations

The pK_a values of ascorbic acid are 4.1 and 11.79 (WEAST, 1982). Basically, the undissociated (H_2A) and monoanionic (HA^-) forms of ascorbic acid predominate in solutions at pH 3.2 and 5.6, respectively. Cupric and ferric ions were tested for catalysis as their nitrate salts,

because, among the inorganic anions, copper (II) forms the least stable complex with the relatively weak Lewis base: nitrate. Sulfate and chloride form more stable complexes with copper, but all of them are relatively weak.

The oxidation of ascorbic acid in the presence of an excess of oxygen (saturation) was assumed to follow a first order reaction (KHAN and MARTELL, 1967a,b; OGATA *et al.*, 1968). The reaction rate can be expressed as

$$- d [H_2A] / dt = k [H_2A] \ldots$$
$$\ldots \text{(proportional to } A_{450} \text{ with Cu(II)-Nc)}$$

where $[H_2A]$ is the concentration of the remaining ascorbic acid during the course of autoxidation, t is the time and k is the first order rate constant which can be calculated from a plot of log $[H_2A]$ *vs* time. It gives a straight line with a slope -k/2.303.

Statistical analysis

The first order rate constant (k) was calculated from the slope of the linear plot of log $[H_2A]$ *vs* time, for m=7 experimental data. The slope and intercept of the line equation: (y = a + bx), found by regression analysis, are b and a, respectively. The standard deviation (s_b) of the slope of these lines (drawn at fixed pH values of 3.2, 4.5 and 5.6) was calculated using the statistics literature (DAVIS *et al.*, 1978) with the aid of the following equations:

$$\Delta = m \Sigma x_i^2 - (\Sigma x_i)^2$$
$$s_y = (\Sigma[y_i - (a+bx_i)]^2 / (m-2))^{1/2}$$
$$s_b = (m s_y^2 / \Delta)^{1/2}$$

where m is the number of measurements at a given pH, x_i and y_i are the corresponding abscissa and ordinate values of the regression-fitted linear equation: (y = a+ bx), s_y is the residual standard deviation of points along the fitted line, and s_b is the standard deviation of the slope (b). The relative standard deviation (RSD) of the slope is given by:

$$RSD = 100 \, s_b/b$$

to reflect the precision of the calculated k values.

Uncatalyzed and copper(II) – or iron(III) ion – catalyzed oxidation

In order to follow the uncatalyzed and Cu(II) or Fe(III) ion–catalyzed oxidation of 0.567 mM ascorbic acid, solutions with cupric or ferric ion levels of 0.157, 0.314, 0.471 and 0.628 μM were studied at 25°C and ionic strength: I=0.1(KNO_3) using Cu(II)-Nc as reagent for the spectrophotometric AA assays. The criteria for choosing room temperature for the kinetic experiments were based on earlier findings that reported that APM may degrade, in part, into benzaldehyde by a Fenton-type reaction caused by Cu(II)+ascorbate at 40°C (LAWRENCE and YUAN, 1996), while at temperatures up to 80°-90°C, aspartame may decompose into aspartyl-phenylalanine and diketopiperazine (PRUDEL *et al.*, 1986). The current reaction was observed for 60 min over a pH range of 3.2-5.6 in acetate buffered solutions with or without APM at levels of 0.1-4 mM. During the first hour, the uncatalyzed autoxidation of ascorbic acid occurred at a conversion ratio of only 1.5%. The transition metal–catalyzed oxidation of ascorbic acid, with respect to time, was evaluated with a compensation for the uncatalyzed reaction. The first-order rate constants were calculated from the slopes of the straight lines by linear regression analysis. The rate increased as the cupric or ferric concentration increased at a given pH. Kinetic curves of log (Abs) *vs* time (min) concerning the oxidation of ascorbic acid in acetate-buffered aspartame solutions are presented in Fig. 3.

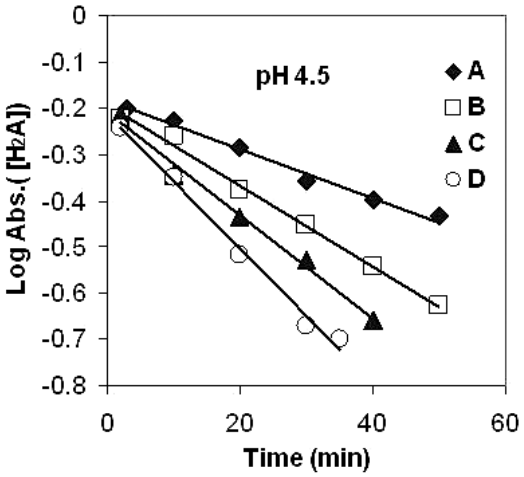


Fig. 3 - Kinetic data reported in accord with first-order reaction kinetics for oxidation of ascorbic acid in acetate-buffered aspartame solutions (2 mM APM) at different Cu(II) concentrations [A:0.157, B: 0.314, C: 0.471, D: 0.628 μM].

Copper-catalyzed AA oxidation in the absence or presence of aspartame

– Effect of pH on the reaction rate of copper-catalyzed ascorbic acid oxidation

The reaction rate of Cu-catalyzed AA oxidation increased as the pH increased from 3.2 to 5.6 in acetate-buffered solution without (Fig. 4a) or with aspartame (Fig. 4b). Since the pK_{a1} of AA is 4.1, acetate-buffered solutions, in the absence of APM, showed a rapid increase of Cu(II) concentration-dependent reaction rates with an increase in pH up to 5.6 (Fig. 4a). This was probably due to an increase in the relative abundance of monohydrogen ascorbate (HA^-) which is thought to be responsible for the formation of the transition species HA-Cu(II)-acetate. However, in the presence of APM, the rate increase with increased pH was observable but it was not so pronounced (Fig. 4b),

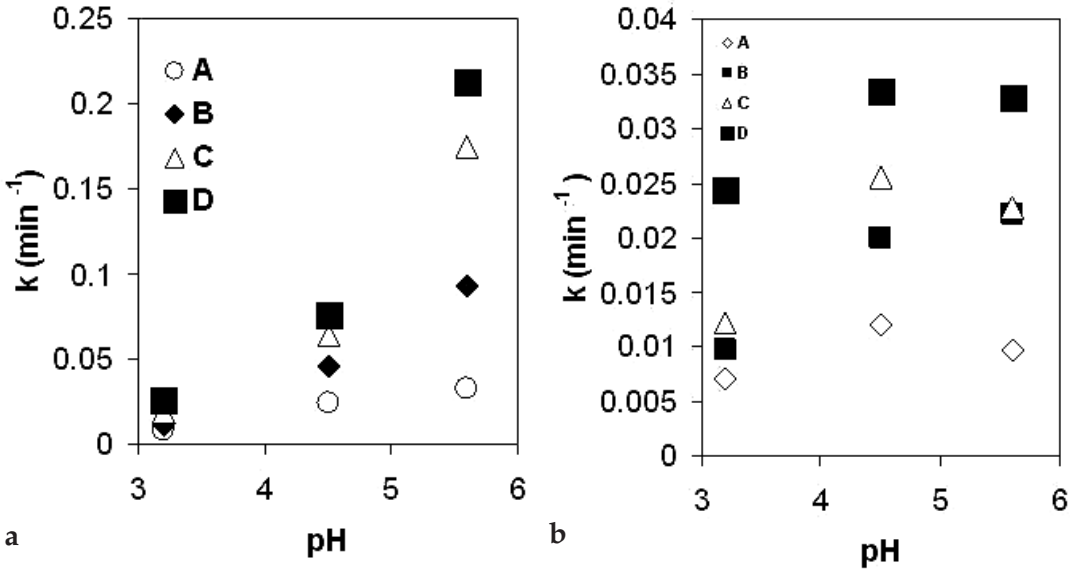


Fig. 4 - Dependence of the rate constant (k) of ascorbic acid oxidation on pH in a) acetate-buffered solutions, Cu(II) concentrations [A: 0.157, B: 0.314, C: 0.471, D: 0.628 μM] and b) acetate-buffered aspartame solutions (2 mM APM), Cu(II) concentrations [A: 0.157 , B: 0.314 , C: 0.471, D: 0.628 μM]. (k was calculated from m=7 experimental data for each fixed pH, with a relative standard deviation of the slope of Log (AA concn.) *vs* time linear curve (RSD) varying between 2.5% and 5.8%, depending on the linear correlation coefficients of the corresponding curves: r^2 between 0.998 and 0.983, respectively. For calculations, see the section 'Kinetic studies, statistical analysis').

Table 1 - Specific rate constants (K) of Cu(II)- aspartame catalyzed oxidation of ascorbic acid at different APM concentrations and pH levels.

[APM] (mM)	pH 3.2 $K \pm s_k$ ($M^{-1}\,min^{-1}$)	pH 4.5 $K \pm s_k$ ($M^{-1}\,min^{-1}$)	pH 5.6 $K \pm s_k$ ($M^{-1}\,min^{-1}$)
0	$(3.69\pm0.11)\times10^4$ r^2= 0.997	$(12.0\pm0.69)\times10^4$ r^2=0.987	$(37.6\pm1.95)\times10^4$ r^2=0.989
0.1	$(3.61\pm0.42)\times10^4$ r^2= 0.960	$(8.90\pm0.49)\times10^4$ r^2=0.988	$(19.1\pm0.89)\times10^4$ r^2=0.991
0.5	$(3.63\pm0.34)\times10^4$ r^2= 0.975	$(7.84\pm0.32)\times10^4$ r^2=0.995	$(11.6\pm0.27)\times10^4$ r^2=0.998
1.0	$(3.72\pm0.38)\times10^4$ r^2= 0.970	$(6.84\pm0.35)\times10^4$ r^2=0.998	$(8.29\pm0.37)\times10^4$ r^2=0.994
2.0	$(3.81\pm0.39)\times10^4$ r^2= 0.979	$(4.44\pm0.12)\times10^4$ r^2=0.998	$(4.79\pm0.25)\times10^4$ r^2=0.994
4.0	$(3.45\pm0.06)\times10^4$ r^2=0.999	$(3.43\pm0.13)\times10^4$ r^2=0.996	$(2.30\pm0.13)\times10^4$ r^2=0.990

s_k = Standard deviation of K values, r^2 = correlation coefficient squared;

K= Specific rate constant independent of concentration, $K = \frac{k_d}{[Cu(II)]}$

because the stable complex-forming APM hindered the formation of the ternary HA-Cu(II)-APM transition species that determined the reaction rate.

– Effect of Cu(II) concentration on the reaction rate of ascorbic acid oxidation

In the following discussion, the symbol k stands for the first-order rate constant, k_d for the difference between the first-order rate constants in the presence and absence of the metal ion, as defined by KHAN and MARTELL (1967a), and K for the specific rate constant, independent of the concentration such that $K = k_d / [Cu(II)]$, as defined by ŞAHBAZ and SOMER (1993). The differences between the first order rate constants in the presence and absence of Cu(II) ion (k_d) were evaluated to describe the rate characteristics. The specific rate constants (K) given in Table 1 were calculated from the slopes of the regression lines plotted as k_d *vs* Cu(II) concentration.

The variations of the k_d values, i.e., equal to K[Cu(II)] with respect to the total Cu(II) concentration at pH=4.5 in acetate buffer alone or acetate-buffered aspartame solutions, are depicted in Fig. 5. The specific rate constants obtained for the Cu(II)-aspartame-catalyzed oxidation reactions at different pH val-

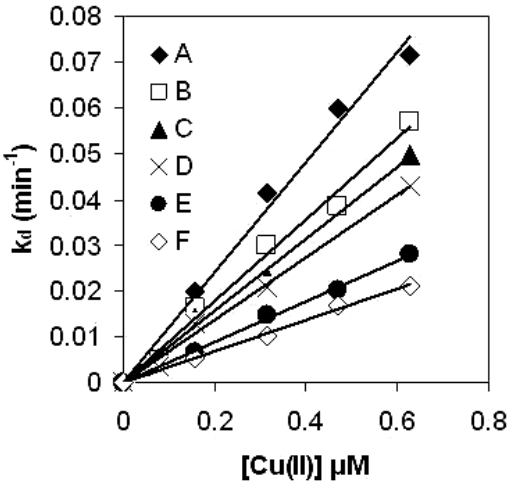


Fig. 5 - Catalytic effect of Cu(II) ion on the oxidation of ascorbic acid in acetate buffer or acetate buffered aspartame solutions [(A: 0, B: 0.1, C: 0.5, D: 1, E: 2, F: 4 mM APM]; k_d = difference between the first- order rate constants in the presence and absence of copper ion, at pH=4.5.

ues are given in Table 1. The specific rate constants (K) of the Cu(II) ion-catalyzed oxidation of ascorbic acid in acetate-buffered aspartame solutions were lower than those in acetate buffer alone at pH=4.5 and 5.6, but were almost unchanged (within tolerable error levels) at pH=3.2. The decrease in specific rate constant values as a function of increasing APM concentration was most significant at pH=5.6, while at pH=3.2, where Cu(II)-APM complexation is negligible, this effect was not observed.

Ascorbic acid autoxidation in pharmaceutical tablets

The initial k values (with 0 and 0.1 mM APM added) for 'redoxon chewable tablets' were significantly less than those of pure standard AA solution (Table 2). This may have been due to the protective action of the original APM content in the tablets. When a critical concentration of APM (0.5 mM) was used, the rate constant reached an almost limiting value beyond which protection did not increase appreciably with concentration. The rate constants for the 'C-plan chewable tablets' were slightly higher than for the redoxon tablets at all the APM concentrations tested, due to the lack of APM in the original formulation of the former. Still, the rate of change of k as the APM concentration changed was smaller for the C-plan tablet solution than for pure AA solution, because the C-plan tablet contained ingredients other than APM that may have limited the AA-protective effect. On the other hand, the original 'redoxon effervescent tablet' solution had a much lower rate constant of AA oxidation ($k=3.59 \times 10^{-3}$ min^{-1}) than the pure AA solution ($k=27.20 \times 10^{-3}$ min^{-1}). This shows that a combination of APM and citrate components of the effervescent tablet significantly stabilized AA. If citric acid was already present in a solution mixture along with APM as a preservative, the AA-stabilizing effect of APM could only emerge when the APM level was in large excess of the Cu(II) level and in an appreciable proportion to AA (e.g., our experimental conditions were AA: 0.567 mM, Cu(II): 0.157 μM, and added APM: 1 mM at pH = 4.5). Moreover, the rate of change of k as APM concentration increased was much slower, probably due to the double-protective effect of the (APM + citrate) combinations (Table 2).

Table 2 - Kinetic study with pharmaceutical tablets* of vitamin C: final concentration of AA in diluted tablet solution and in pure solution were equimolar (0.567 mM) at a Cu(II) concentration of 0.157 μM and pH = 4.5.

	k (min^{-1})		
Added [APM] (mM)	Redoxon soln.	C-Plan soln.	Pure standard AA soln.
0	22.72×10^{-3} $r^2=0.932$	24.49×10^{-3} $r^2=0.991$	$27,20 \times 10^{-3}$ $r^2=0.982$
0.1	20.45×10^{-3} $r^2=0.972$	21.79×10^{-3} $r^2=0.990$	24.95×10^{-3} $r^2=0.960$
0.5	15.68×10^{-3} $r^2=0.946$	17.86×10^{-3} $r^2=0.786$	15.03×10^{-3} $r^2=0.980$
1.0	14.00×10^{-3} $r^2=0.914$	15.29×10^{-3} $r^2=0.984$	13.83×10^{-3} $r^2=0.992$

*The solution obtained – by double filtration – from effervescent tablets at the same AA concn. (0.567 mM) was turbid, so only the initial rate constant (without APM addition) could be measured: $k_{initial}=3.59 \times 10^{-3}$ min^{-1} ($r^2=0.810$). Instead of this tablet solution, a synthetic AA solution was prepared by assuming 10% tablet mass of $NaHCO_3$ and citric acid, finally diluted to 0.567 mM AA: its AA oxidation rate constant, k, decreased from an initial value of 16.0×10^{-3} ($r^2=0.85$) with no APM to 13.9×10^{-3} min^{-1} ($r^2=0.91$) with 1.0 mM APM added.

One of the effervescent tablet ingredients, bicarbonate, does not have any effect on the catalytic oxidation of AA at pH 4.5. Since sorbitol does not apparently have Cu-chelating functional groups, it may not exert a chemical effect, but may exert a physical effect at very high concentrations due to decreased water activity, e.g., induced by a similar sugar, sucrose (HSIEH and HARRIS, 1993). Citric acid is the only ingredient that may affect the catalytic oxidation *via* Cu(II)-complexation, along with APM. At pH

4.5, APM (H_2Asp) is mainly in the $HAsp^-$ state and 65 % and 35 % of the citric acid (H_3Cit) are in the H_2Cit^- and $HCit^{2-}$ states, respectively. The stability constant of 1:1 complex, log K_1, for Cu(II)-$HAsp^-$ is 6.1, while for Cu(II)-$HCit^{2-}$, log K_1 is 4.35. Considering the proportion of $HCit^{2-}$ available at pH 4.5, it was apparent that the primary AA protective action of the effervescent tablet of vitamin C depends on its APM content due to its stronger Cu(II)-chelating ability.

In an overall evaluation, the level of AA protection measured in the aqueous solution of pharmaceutical vitamin C tablets, expressed as the rate constant (k) of AA catalytic oxidation and its rate of change with increased APM concentration, verified that AA was protected from oxidation either in APM- containing or APM- added formulations (Table 2). If there were other tablet ingredients (e.g., citrate) capable of complexing Cu(II), the catalytic oxidation of AA proceeded much less slowly due to the double protective effect of (APM + citrate). The critical concentration of APM, beyond which AA protection could not be enhanced, was in sufficient excess of Cu(II) and similar to the AA levels.

Fe(III)-catalyzed AA oxidation in the absence or presence of aspartame

As in the case of the Cu(II) ion–catalyzed oxidation, the experimental results for Fe(III) indicated a first-order reaction with respect to the ascorbic acid concentration. At pH 4.5, a linear variation of rate with the total concentration of Fe(III) ions was observed, indicating the observed catalytic behavior of Fe(III) ions.

Ferric ions undergo extensive hydrolysis even at low pH values. The concentration of ferric ions was not corrected for hydrolysis considering the hydrolysis constants given by HEDSTROM (1953). The successive hydrolysis equilibria with hydrolytic equilibrium constants K_{h1} and K_{h2} are represented by the following equations:

$Fe^{3+} + H_2O \leftrightarrow Fe(OH)^{2+} + H^+$	$K_{h1} = [Fe(OH)^{2+}]\,[H^+] / [Fe^{3+}]$
$Fe(OH)^{2+} + H_2O \leftrightarrow Fe(OH)_2^+ + H^+$	$K_{h2} = [Fe(OH)_2^+]\,[H^+] / [Fe(OH)^{2+}]$

If $[Fe(III)]_t$ represents the total concentration of metal species, then the concentration of the unhydrolyzed ferric cation, Fe^{3+}, may be calculated using the following equation:

$$[Fe^{3+}] = [\,Fe(III)]_t \,(1 + K_{h1} / [H^+] + K_{h1} K_{h2} / [H^+]^2)^{-1}$$

The variation of rate constants at 25°C with the concentration of total ferric ion at pH= 4.5 is indicated in Fig. 6. The specific rate constants given in Table 3 were calculated from the slopes of the straight lines in Fig. 6. The specific rate constants of Fe(III)-catalyzed oxidation (Table 3) at pH=4.5 were at least one order of magnitude lower than those of Cu(II) (Table 1). This is in accord with the findings of HSIEH and HSIEH (2000) who found that at pH≥5, Fe(III) is reduced very slowly by AA. The observations regarding the lower rate constants achieved with Fe(III) both in the absence and presence of APM, and the unexpected slight increase of specific rate constants with APM concentration (Table 3) may have been due to the complexity and poor reversibility of Fe(III) hydrolysis at pH≥4. Preliminary experiments with Fe(III) at low pH gave somewhat inconsistent results (unreported data), because the pK_{a1} of APM was 3.1 which probably prevented complexation.

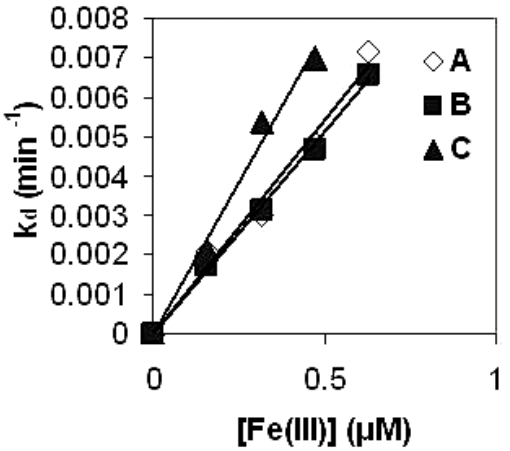


Fig. 6 - Catalytic effect of Fe(III) ion on the oxidation of ascorbic acid in acetate buffer or acetate buffered aspartame solutions [(A: 0, B: 0.1, C: 0.2 mM APM); k_d = difference between the first- order rate constants in the presence and absence of ferric ion, at pH=4.5.

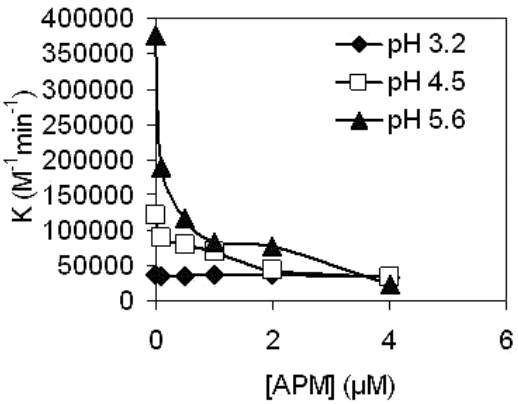


Fig. 7 - Dependence of the specific rate constants, K, on aspartame concentrations at different pH values, in acetate buffer for the Cu(II)- catalyzed oxidation of ascorbic acid.

General features of transition metal-catalyzed AA oxidation in the presence of APM

The observed effects of pH, cupric and ferric ion concentrations and aspartame concentration on the rate of ascorbic acid oxidation can be summarized as follows:

Table 3 - Specific rate constants (K) of Fe(III)- aspartame catalyzed oxidation of ascorbic acid at different APM concentrations and at pH: 4.5.

[APM] (mM)	K±s_k (M^{-1} min^{-1})
0	(1.09 ± 0.08) x 10^4 r^2=0.983
0.1	(1.02 ± 0.03) x 10^4 r^2=0.997
0.2	(1.54 ± 0.13) x 10^4 r^2 = 0.985

s_k = Standard deviation of K values, r^2 = correlation coefficient squared;
K= Specific rate constant independent of concentration, $K = \frac{k_d}{[Fe(III)]}$

1) The rate constants increased as the Cu (II) or Fe (III) ion concentrations increased (See Figs. 5, 6).

2) The rate constants of copper-catalyzed oxidation increased as the pH was increased to 5.6 in acetate-buffered solutions with or without aspartame (See Figs. 4a,b); the increase with respect to pH was much more pronounced in the absence of aspartame.

3) Specific rate constants of copper-catalyzed oxidation decreased as the aspartame concentration increased, at pH 4.5 or 5.6. These constants did not significantly change with respect to APM at pH 3.2 due to a weaker Cu(II)-APM complexation (Fig. 7).

The cumulative stability constants (Log β_n) of the Cu(II)-acetate complexes were 2.16 and 3.2 for mono- and bis-complexes, respectively (LURIE, 1978). The highest reaction rate with Cu(II)-acetate was achieved at pH=5.6 (Fig. 4a). However, the reaction rates decreased in acetate buffered aspartame solutions in the same pH range. Free Cu^{2+} ion (such as $[Cu(H_2O)_4]^{2+}$) and the weak Cu(II)-acetate complex catalyzed AA oxidation to

a greater extent (IMER *et al.*, 2003) than the relatively stable Cu(II)-aspartame complex. The stability constants (Log β_n) of the Cu(II)-aspartame complexes were 6.097 and 10.84 for CuL^+ and CuL_2, respectively, (KHOLEIF and ANDEREGG, 1997) rendering cupric ion complexation with aspartame predominant in acetate-buffered solutions.

The corresponding concentrations of free cupric ion may only be important in the Cu(II)-acetate system where the ratio of free [Cu^{2+}] to total [Cu (II)] was about 1/1.22 for the total Cu (II) ion and buffer concentrations studied. In the aspartame system, the free Cu^{2+} concentration was negligible in a solution containing an excess (more than 100–fold) of a stable complex-forming ligand like aspartame.

These findings were in accord with those of the literature. For example, KHAN and MARTELL (1967b) established that the catalytic activity of cupric ion was greater than that of ferric ion toward the oxidation of monohydrogen ascorbate (HA^-). However, in the acidic pH range where neutral H_2A species predominate, the catalytic order may differ. The rate constants of Fe(III)-catalyzed oxidation of AA at pH 4.4 indicated a much slower reaction than that observed in 0.2 M H^+-containing medium. The nature of the Fe(III)–associated ligands is important in the inner-sphere electron transfer with HA^- which probably indicates the importance of a ternary transition complex involving Fe(III)-ligand-monohydrogen ascorbate (XU and JORDAN, 1990; BANSCH *et al.*, 1991).

KHAN and MARTELL (1967b) proposed the following mechanism for Cu(II)- or Fe(III) chelate (ML^{n+})-catalyzed oxidation of ascorbic acid (H_2A):

$$H_2A \leftrightarrow HA^- + H^+$$
$$HA^- + ML^{n+} \leftrightarrow MHLA^{(n-1)+}$$
$$MHLA^{(n-1)+} \rightarrow MHLA^{\cdot(n-1)+}$$
$$MHLA^{\cdot(n-1)+} \xrightarrow{\text{rapid}\rightarrow} A^{\cdot-} + ML^{(n-1)+}$$
$$A^{\cdot-} + ML^{n+} \xrightarrow{\text{rapid}\rightarrow} A + ML^{(n-1)+}$$

where the catalyst ML^{n+} may be regenerated by oxidation of $ML^{(n-1)+}$ with dissolved O_2 (here, H_2A denotes ascorbic acid, and A, the 2-e oxidized product, dehydroascorbic acid). In this multi-step process, the slow (rate-determining) step is the formation of $MHLA^{(n-1)+}$ ternary transition complex from monohydrogen ascorbate (HA^-) and Cu(II)- or Fe(III)- chelate (ML^{n+}). If the binary M-L chelate is too stable, the ternary transition complex may not form. Likewise, if negative charge accumulates on the M-L chelate (e.g., FeY^- or CuY^{2-}, where Y^{4-} is the EDTA anion), then the ternary transition complex may not form due to electrostatic repulsion between HA^- and the binary metal chelate. Less rigid bonding for the ternary transition complex results in easy deformation of this complex upon electron transfer. Thus, KHAN and MARTELL (1967b) hypothesized that "the ascorbic acid autoxidation rate in the presence of Cu(II)- or Fe(III)- chelate catalyst decreases with increasing stability and increasing negative charge on the M-L chelate". Their hypothesis was in accord with their observation for the linear correlation between the stability of the metal chelate and its catalytic activity. Again in accord with this hypothesis, the copper complexes of Cu(II)-phenanthroline, Cu(II)-dipyridyl (KHAN and MARTELL, 1967b) and Cu(II)-thiourea (ZHU, *et al.*, 2002) were completely inactive in catalyzing ascorbic acid autoxidation. Thus, as the stability of Cu(II)-L increases, the tendency of CuL to form a mixed ligand chelate with hydrogen ascorbate (HA^-) decreases (KHAN and MARTELL, 1967b). Although the findings of HSIEH and HARRIS (1991) on the role of aspartame in the Cu(II)-catalyzed ascorbic acid oxidation system were somewhat contradictory to the aforementioned rule, our findings on the possible role played by APM in the copper-catalyzed system conformed perfectly with the conclusions reached by KHAN and MARTELL (1967b).

Another application of the rule that ligands with strong complexing groups sequester traces of transition metal ions responsible for catalytic oxidation of AA is the development of AA substitutes, sodium- and magnesium- ascorbyl phosphates (Na- or Mg-AP), in the pharmaceutical industry for use in cosmetic products (topical formulations). They are much more stable hydrophilic ascorbic acid derivatives. For such products, it is claimed that the phosphate group neighboring the cyclic ring of Na- or Mg-AP protects the enediol system of the ascorbate moiety from oxidation (SPICLIN *et al.*, 2001). In addition the chelating complexation ability of the associated PO_4^{3-} group of these ascorbyl phosphates may also constitute a sink for traces of transition metal catalysts that would render the compound an antioxidant only when hydrolyzed to AA by enzymes present in the skin.

Naturally there is the risk of prooxidant action of AA in preference to its conventional antioxidant role under certain experimental conditions, especially when there is an appreciable concentration of the free transition metal ion. Ascorbic acid-metal ion interactions involve redox reactions including: (i) activation of molecular oxygen leading to oxidation of endogenous aromatic moieties; (ii) the reduction reactions of Fe(III) to Fe(II) or Cu(II) to Cu(I) facilitating their involvement in the activation of peroxides (i.e., the Fenton reactions: $Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + Fe(III) + OH^-$ or $Cu(I) + H_2O_2 \rightarrow {}^{\bullet}OH + Cu(II) + OH^-$); (iii) metal ion catalysis of the oxidation of ascorbic acid with concomitant formation of hydrogen peroxide (H_2O_2) and potential hydroxyl radical ($^{\bullet}OH$) generation (FISCHER and NAUGHTON, 2004). Thus the contradictory results of HSIEH and HARRIS (1991) on the role of APM in Cu-catalyzed oxidation of AA may have been due to different experimental conditions that promoted the prooxidant effect of AA. Although these authors used approximately the same amount of APM (0.06% solution) as we did in our experiments, they used 10-100 times more Cu^{2+} (1-10 ppm Cu) and 10 times more AA (0.1% solution). Thus, HSIEH and HARRIS (1991) calculated that about 1/3 of their total Cu(II) ion was uncomplexed under their experimental conditions for a total Cu concentration of 10 ppm. In contrast to our experimental design where practically all Cu(II) was bound to APM in a stable complex, an excess of uncomplexed Cu(II) may have easily induced the prooxidant effect of AA, which may have led these authors to conclude that "APM increased the rate of Cu-catalyzed AA oxidation in all tested solutions at 33°C" (HSIEH and HARRIS, 1991). LAWRENCE and YUAN (1996) showed that in weakly acidic solution, APM, in the presence of AA + transition metal catalyst under aerobic conditions, can produce benzaldehyde *via* free radical attack on APM. HSIEH and HARRIS (1991) reported that they had expected the strongly Cu-complexing ligand APM to reduce the AA oxidation rate. However, the complexes formed between Cu(II) and APM seemingly facilitated O_2 activation reactions with AA molecules. What they reported to be a faster AA degrading Cu-APM complex may actually have been a partial degradation of APM at relatively high Cu(II) and AA concentrations which led to a cycle of free radical attacks by Fenton-type reactions.

The Cu(II)-chelates formed as a function of pH and their relative stabilities must be considered when evaluating catalytic acitivity. Two mechanisms have been considered for the Cu(II)- aspartame-catalyzed oxidation of ascorbic acid: (1) a dissociation mechanism, which may be due to the free Cu^{2+} ion in equilibrium with the chelate compound and (2) the direct participation of Cu(II)-chelates in an electron-transfer process (MARTELL, 1982). The first assumption was previously shown to be invalid for relatively strong metal chelates (KHAN and MARTELL, 1967b), and according to the second mechanism, the copper

chelate with the aspartame ligand may form a ternary transition complex with the ascorbic acid monoanion [HA^-] which may be converted to the Cu(I)-chelating aspartame complex and the radical product of ascorbic acid [•HA] in a slow reaction (IMER *et al.*, 2003). The ascorbyl radical responsible for the reaction with molecular oxygen (ascorbic acid itself being converted to dehydroascorbic acid) (ŞAHBAZ and SOMER, 1993) was actually detected in the Cu(II)-histidine-AA reaction system by ESR techniques (SCARPA *et al.*, 1996). Our finding on the inhibitory role of aspartame in copper–catalyzed AA autoxidation is important because of the cooperative roles of antioxidants in preserving the nutritional value of food. For example, the role of glutathione (GSH) in inhibiting Cu(II)-catalyzed autoxidation of AA has been attributed to 1:1 chelate formation of GSH with Cu(I) in which the copper needed as catalyst is sequestered (OHTA *et al.*, 2000). This cooperative antioxidant action of GSH, along with that of AA, may be explained by a rapid reduction of the thiyl radical of GSH by AA and by the GSH dose-dependent delay of hydroxyl radical formation in the course of Cu(II)-catalyzed oxidation. This cooperative role of GSH and AA may be effective *in vivo*, and both APM- and AA- containing beverages may exhibit a similar synergistic potency *in vitro*. A similar finding in the literature is associated with the combination of ascorbate and α-tocopherol that produces a synergistic effect in inhibiting oxidation, where AA reduces α-tocopheroxyl radicals rapidly in membranes to generate α-tocopherol which may inhibit α-tocopheroxyl radical-mediated propagation (NIKI *et al.*, 1995).

CONCLUSIONS

The rate equation of AA oxidation in the presence of cupric and ferric ions as catalyst at pH levels of 3.2, 4.5 (suitable for most carbonic acid-containing drinks) and 5.6 was found to be first order with respect to AA. At a given pH, AA oxidation rate constants decreased with increasing APM concentration for a fixed Cu(II), and increased with increasing Cu(II) or Fe(III) concentration for a fixed APM level. The dependence of the AA oxidation rate on the studied parameters suggests that the mechanism may involve the formation of a ternary transition complex between monohydrogen ascorbate and Cu(II)-aspartame chelates. The catalytic activity of copper(II) was more affected than that of iron (III) in APM solutions. This was probably due to the stronger copper-complexing ability of aspartame in the pH range used (though the stabilities of Fe (III)-APM complexes have not been well documented in the literature). Free Cu^{2+} ions (such as $[Cu(H_2O)_4]^{2+}$) and the weak Cu(II)-acetate complex were shown to catalyze AA oxidation to a greater extent than the relatively stable Cu(II)-aspartame complex. This finding supports the conclusion of KHAN and MARTELL (1967b), i.e., as the stability of a Cu(II) complex increases, its catalytic activity as a mediator of electron-transfer in AA oxidation decreases. Since APM is a widely used sweetener in beverages, it may help protect their AA values by slowing transition metal-catalyzed oxidation. The cooperative action of antioxidants is reported in the literature for AA+glutathione and AA+α-tocopherol pairs (NIKI *et al.*, 1995). APM also helps stabilize transition metal ion+AA mixture solutions which would otherwise yield Fenton-like reactions causing hydroxyl radical-induced degradations. Therefore APM could be used to conserve foods and beverages. This is important, because antioxidants such as ascorbic acid in food may act as pro-oxidants by reducing transition metal ions like Fe(III) and Cu(II) to the lower oxidation state and make the strongly oxidizing hydroxyl radical generation catalytic (HUANG *et al.*, 2005). The AA-protec-

tive effect of APM by slowing metal-catalyzed oxidation of AA should be exploited in pharmaceutical products to develop formulations containing vitamin C with relatively high water activity and weakly acidic-to-neutral pH. For the first time, ascorbic acid degradation has been spectrophotometrically monitored with a copper(II)-neocuproine assay without being influenced by aspartame or many other organic compounds that normally interfere with conventional UV-spectrometric assays.

ABBREVIATIONS

AA	Ascorbic acid
APM	Aspartame
ESR	Electron spin resonance
HA^-	Monoanionic form of ascorbic acid
H_2A	Undissociated ascorbic acid
GSH	Glutathione
Nc	Neocuproine: 2,9-dimethyl-1,10-phenanthroline
UV	Ultraviolet-Visible
K_a	Acidity constant
β_n	Cumulative complex formation constant
s_b	Standard deviation of the slope of a line
RSD	Relative standard deviation

ACKNOWLEDGEMENT

R. Apak would like to express his gratitude to the Istanbul University Research Fund, Bilimsel Arastirma Projeleri Yurutucu Sekreterligi, for the funding of Project YOP-4/27052004, and to the State Planning Organization of Turkey for the Advanced Research Project of Istanbul University (2005K120430). Gratitude is also expressed for the opportunity to present a part of this work at an international meeting supported by the Research Fund of Istanbul University (Bilimsel Arastirma Projeleri Yurutucu Sekreterligi) under project UDP-825/19072006.

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Paper received February 21, 2007 Accepted July 17, 2007

PAPER

DETERMINATION OF SOLUBLE SOLIDS CONTENT IN FUJI APPLES BASED ON NEAR INFRARED SPECTROSCOPY AND ARTIFICIAL NEURAL NETWORKS

DETERMINAZIONE DEL CONTENUTO DI SOLIDI SOLUBILI NELLE MELE FUJI ATTRAVERSO LA SPETTROSCOPIA NEL VICINO INFRAROSSO E RETI NEURALI ARTIFICIALI

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ABSTRACT

Soluble solids content of intact apples has been predicted by artificial neural networks from their near-infrared spectra using an acousto-optic tunable filter. The principal components scores of spectral data were used as network input. The degree of approximation was performed to prevent over-fitting during network training. The optimum network topology was established with five input nodes, three hidden nodes, a learning coefficient of 0.2 and a momentum of 0.8. The corresponding best model had

RIASSUNTO

È stato determinato il contenuto in solidi solubili di mele integre, attraverso reti neurali artificiali, dai loro spettri nel vicino infrarosso utilizzando un filtro acusto-ottico modulabile. I posizionamenti delle componenti principali dei dati spettrali sono stati utilizzati come input nelle reti. Il grado di approssimazione è stato fissato per prevenire l'over-fitting durante la messa a punto delle reti. In questo lavoro la topologia ottimale delle reti è stata fissata con cinque nodi di input, tre nodi na-

- Key words: Apple, Artificial neural networks, degree of approximation, near infrared, principal components, soluble solids content -

a correlation coefficient (r) of 0.877, a relative standard deviation of training (RSDc) of 7.59% and a relative standard deviation of prediction (RSDp) of 8.38%. In addition, the network structure was more important for establishing the model than the parameters which did not have significant effect on the network. Compared with partial least squares regression after spectral pre-treatment (r=0.867, RSD_C=7.86%, RSD_P=8.76%), artificial neural networks had a little better prediction ability. Results showed that the methodology was satisfactory for rapid analysis of internal fruit quality.

scosti, un coefficiente di apprendimento di 0,2 ed un momento di 0,8. Il corrispondente modello ottimizzato aveva un coefficiente di correlazione (r) di 0,877, una deviazione standard relativa di training (RSDc) di 7,59% ed una deviazione standard relativa di predizione (RSDp) di 8,38%. Inoltre, nello stabilire il modello, la struttura della rete è stata più importante rispetto ai parametri che hanno dimostrato di non presentare un effetto significativo. Paragonate alla regressione dei minimi quadrati parziali, dopo pretrattamento degli spettri (r=0,867, RSDC=7,86%, RSDP=8,76%), le reti artificiali neurali hanno dimostrato di avere una abilità predittiva migliore. I risultati hanno dimostrato inoltre che la metodologia era soddisfacente per effettuare analisi rapide della qualità interna del frutto.

INTRODUCTION

The near infrared (NIR) spectroscopy region of the electromagnetic spectrum contains information concerning the relative proportions of C-H, N-H and O-H bonds, which are the primary structural components of organic molecules (OSBORNE *et al.,* 1993). When a fruit or vegetable is exposed to light, about 4% of the incident light is reflected at the outer surface, causing specular reflectance, and the remaining 96% of the incident energy is transmitted through the surface into the cellular structure of the substance where it is scattered by the small interfaces within the tissue or absorbed by cellular constituents (BIRTH, 1976). The diffuse reflection spectrum is altered by different absorbances of various wavelengths before exiting and therefore contains useful sample information. Analysis of NIR diffuse reflectance spectra provides quantitative determinations of internal fruit quality properties (LAMMERTYN *et al.,* 1998; ZUDE *et al.,* 2002).

With the advantages of rapid and nondestructive measurement, NIR is a well-established technique for internal quality analysis of fruit (KAWANO *et al.,* 1992; SLAUGHTER, 1995). Multivariate linear calibration methods, such as multiple linear regression (MLR) (BLANCO *et al.,* 1994), principal component regression (PCR) (WOLD *et al.,* 1987) and partial least squares regression (PLSR) (HAALAND *et al.,* 1988), are widely used in NIR determination. These approaches successfully assume a linear relationship between the target analytical parameters of samples and the absorbance. Nevertheless, in complex nonlinear systems, the classical multivariate calibration techniques usually fail to build models.

In fact, the chemical nature of apples always presents nonlinear quality which leads to large prediction errors. Under

external ambient conditions (pressure, temperature, etc.), the different kinds of spectral interactions can shift absorption bands and result in intrinsic nonlinearity (BERTRAN *et al.,* 1999).

Currently, several methods have been proposed to calibrate nonlinear data, such as pre-treatment (DHANOA *et al.,* 1994) and nonlinear calibration techniques (SEKULIC *et al.,* 1993). However, pre-treatments such as wavelength selection and transformation resolve slight nonlinearities. With the advantage of modelling severe intrinsic nonlinearities, the nonlinear calibration can establish robust nonlinear multivariate calibration.

Artificial neural networks (ANN) (ZUPAN and GASTEIGER, 1991; NAES *et al.,* 1993) have been successfully applied to analyze spectroscopic data to overcome nonlinearity. The potentials of ANN are its anti-jamming, anti-noise and robust nonlinear transfer ability. The spectroscopic training using ANN has recently appeared in the literature. LONG *et al.* (1990) used NIR to determine the protein content in wheat, and found that ANN gave superior performance for spectroscopic calibration due to component interactions and nonlinear instrumental response functions. SEVERCAN *et al.* (2001) used neural networks to estimate the secondary structure of protein from Fourier transform infrared (FT-IR) spectra and determined that the methodology was effective. YANG *et al.* (2003) reported that ANN gave better results than PLSR when the data were nonlinear, whether the nonlinearity was caused by band position changes or a nonlinear relationship between peak height and concentration. MARENGO *et al.* (2004) concluded that FT-IR spectroscopy and back-propagation artificial neural networks (BP-ANN) models can be properly employed for monitoring the polyesterification of dicarboxylic acids with diols by predicting the acid and hydroxyl numbers directly along the process line. DOU *et al.* (2005) demonstrated that it was able to build ANN models of first-derivative spectra for determination of two components of compound paracetamol and diphenhydramine hydrochloride powder on NIR spectroscopy.

In this work, multi-layer feed-forward neural networks with the error back-propagation (BP) learning rule was applied to determine the soluble solids content (SSC) in intact apples from their NIR spectra. The network topology is discussed, and namely, the number of nodes in the input and hidden layers was optimized. Moreover, the principal components scores were chosen as nodes of input layer for reducing the large number of data to a smaller set of orthogonal variables. In addition, noise and random error in the original data would be excluded by use of these scores (DOU *et al.,* 2006). The selection procedure of ANN parameters (such as training function, learning coefficient, momentum, number of iterations, etc.) was the main problem resolved. In the network evaluation, the degree of approximation was employed to avoid over-fitting (DOU *et al.,* 2004). Finally, compared with the ANN results, the PLSR models based on three spectral pre-treatment (multiple scatter correction, first derivative and second derivative) were established.

MATERIALS AND METHODS

Instrument

The experimental spectra were measured from an acousto-optic tunable filter (AOTF) spectrometer (Sunshine Optics Technologies Co. Ltd, Tianjin, China). The instrument consists of a standard NIR configuration including a wide band light source (50W/220V) tungsten halogen lamp, AOTF, InGaAs detector, etc. Furthermore, it has a spectral resolution of less than 1 nm, a signal-to-noise ratio of 10000/1, and a wavelength accuracy of ±0.5 nm.

Samples

A total of 93 Fuji apples were obtained from a local supermarket, and placed in an air-conditioned room at 25 °C for a few hours before testing. They were used to establish a relationship between diffuse reflection spectra and the SSC of intact apples. Sixty-seven of these apples were used for the training models, and the other 26 apples were used for SSC prediction.

Diffuse reflectance measurements

Apple samples were placed in the fruit holder with the stem-calyx axis in the horizontal position. For each apple, four diffuse reflection spectra were taken at four equidistant positions along the equator, avoiding any obvious surface defects. A bifurcated optical fiber was used. The light was guided to the sample with a source optical fiber which contacted the sample and was transmitted through the surface into the tissue of the apple. The diffuse reflected light from the internal tissue through the optical fiber detector was collected by an InGaAs detector in the AOTF-NIR instrument, which had a spectral range of 1050-1650 nm. The mean spectrum of each apple was obtained by averaging the spectra collected at the four positions around the apple. During the procedure, after every fifteen apples, the standard reference spectra used in the calculation of absorbance for each sample were obtained from a $BaSO_4$ block with a 30 mm diameter and a 5 mm high cylinder. Each spectrum was converted from reflectance (R) to absorbance (log (1/R)). Auto-scaled data were studied for ANN.

SSC measurement

According to the method of the National Standard of China (GB12295-90) for SSC measurement, the SSC was estimated with a hand-held digital refractometer (WYT-4, Optical Instrument Co., Quanzhou, China). Juice was obtained by squeezing a portion of the flesh removed from the NIR sampling site with a 1.5 cm cork borer, and the °Brix was recorded. The SSC of each apple was the mean value of four measurements from the four positions of the spectra collection. The statistical data of SSC are listed in Table 1.

Method of ANN algorithms

All the neural networks used in this paper were fully-connected feed-forward networks with the BP algorithm (DESPAGNE and MASSART, 1998). The hidden layer consisted of nodes with a hyperbolic tangent function which facilitated nonlinear fitting and an output layer with linear function. Principal component analysis (PCA) is a data mining technique in multi-elements statistics. On the premise of reserving major spectral information, PCA can use fewer new variables instead of original ones to figure out the difficulty of analysis incapable as superpositioning of spectral bands (HE *et al.*, 2007). In this research, PCA was used to transform the large amount of original correlated variables in a smaller set of principal components which were then used as network inputs instead of original data. All the networks had only one output node in the output layer for one parameter (SSC) to be determined. The optimal network architecture was investigated by the different number of input

Table 1 - Statistical data of SSC of 93 samples.

Sample Indexes	Calibration set	Prediction set
No.[a]	67	26
Mean/°Brix	13.2	12.8
Range/°Brix	9.5-18.0	10.9-15.9
Std[b]/°Brix	2.00	1.26

[a] number of samples
[b] standard deviation.

and hidden nodes. Afterwards, the network performance also depended on the training method. The BP-ANN with adaptive learning coefficient and momentum was used to train the network. These performances were implemented in MATLAB 7.0 using the Neural Network Toolbox (DEMUTH and BEALE, 1998).

Model evaluation

During ANN model training, it is a big problem to have over-fitting. To minimize the error of the training set or the monitoring set is not the reliable criterion for optimizing the network, for the error of prediction set may be not at the minimum. This kind of network is unsteady when it is used to predict an unknown sample. To avoid this kind of situation, a new evaluation criterion of the network, the degree of approximation (D_a), was employed (DOU *et al.*, 2004). The criterion is defined by equations (1) and (2)

$$E_a = (n_l/n)e_l + (n_c/n)e_c + |e_l \quad (1)$$

where E_a is the error of the approximation; e_l and e_c are the relative standard errors of training set and monitoring set, respectively; n_l and n_e are the sample numbers of training set and monitoring set, respectively; n is the whole number of training samples; n_l/n and n_c/n are the weights contributed to the E_a by training set and monitoring set, respectively. A new index is defined in equation (2).

$$D_a = c/E_a \quad (2)$$

where D_a is the degree of approximation and c is a constant by which D_a is adjusted to get a good chart. It is very obvious that the smaller is E_a and the larger is D_a, the better ANN models can be obtained. Therefore, the effects of both training set and monitoring set are considered in this evaluation criterion.

The quality of the training model was quantified by the correlation coefficient (r), relative standard deviation of training (RSD_C) and the relative standard deviation of prediction (RSD_P). A good model should have a small RSD_C, a small RSD_P and a high r. The parameters describing the performance of the training model are in the forms:

$$r = \sqrt{1 - \frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{N}(y_i - y_{mc})^2}} \quad (3)$$

$$RSD_C = \sqrt{\frac{1}{N-P-1}\sum_{i=1}^{N}(y_i - \hat{y}_i)^2} \Big/ y_{mc} \times 100\% \quad (4)$$

$$RSD_P = \sqrt{\frac{1}{N_p}\sum_{i=1}^{N_p}(y_{i_p} - \hat{y}_{i_p})^2} \Big/ y_{mp} \times 100\% \quad (5)$$

where N is the number of samples in the training model; y_i is the actual SSC of sample i in the training set; $\hat{y}_i$ is the SSC predicted by the model in the training set; P is the number of latent factors used in the training model; y_{mc} is the mean of all samples SSC in the training set; N_p is the number of samples in the prediction set; y_{i_p} is the actual SSC of sample i_p in the prediction set; $\hat{y}_{i_p}$ is the SSC predicted by the model in the prediction set; and y_{mp} is the mean of all samples SSC in the prediction set.

RESULTS AND DISCUSSION

While being trained, the modifications of the network input nodes, hidden nodes, momentum, learning coefficient and iteration numbers were made by the BP of the error and the D_a. The training set was used to train the network, and the monitoring set was used to avoid over-fitting. The maximal D_a was used to determine the network topology parameters. A non-negligible part of this methodology was dedicated to to-

pology optimization. In order to train an ANN with a high ability of generalization on SSC determination, networks were trained under a wide range of parameter settings.

Determination of number of input and hidden nodes

The number of input and hidden nodes determines the complexity of the neural network, which affects the convergence speed, noise inertness and neural network prediction ability. On the other hand, the network cannot be properly trained at all with too few nodes of the two layers. The number of nodes in the input and hidden layers should be optimized.

In this research, principal components (PCs) were used for the input nodes of the BP-ANN. A few of PCs may not represent enough information of the samples while excessive PCs may include too much noise that is a disadvantage for modeling. Therefore, the selection of the number of PCs was important for the nonlinear regression. Accumulative variance percentage (AVP) was applied to compute the number of PCs and the PCs were obtained by the use of nonlinear iterative partial least squares (NIPALS). The different number of input nodes, namely, the different AVP was changed to PCA (Table 2).

The proper number of hidden nodes helped to improve the prediction performance and the ability of noise inertness. However, there was no theorem to choose the number of nodes, which was mostly determined by experience and experiment. In this research, the number of hidden nodes was initialized by two experience functions. The formulas were as follows (ZHANG, 1993; XU and SHAO, 2004)

$$n_1 = \sqrt{n+m} + a \quad (6)$$

$$N/TW \geq 1 \quad (7)$$

where n is the number of input nodes; m is the number of output nodes; a is the constant between 1 and 10; N is the number of samples; and TW is the total number of weights. According to the PCs number (i.e. input nodes) and equations (1) and (2), it was decided that the number of hidden nodes was from 3 to 15. Under different network structures, BP-ANN was trained. Fig. 1 shows the results of training. Compared with each PC, the RSD_C was reduced and the RSD_P was increased gradually, while the number of hidden nodes increased. The results in Fig. 1 show that the number of input and hidden nodes has a great effect on the predictive results. However, it was difficult to determine the optimum topology from Fig. 1. Moreover, there were difficulties in estimating whether the models were over-fitting and overtraining.

The proper number of nodes in the input and hidden layers for training set was investigated by comparing the D_a for each network. Fig. 2 presents the curve of D_a under different input and hidden nodes, and shows the D_a of different network structures. When input and hidden

Table 2 - The number of PCs under different AVP.

AVP [a](%)	99.900	99.950	99.970	99.990	99.993	99.997
Number of PCs[b]	3	4	5	6	7	8

[a] accumulative variance percentage
[b] principal components.

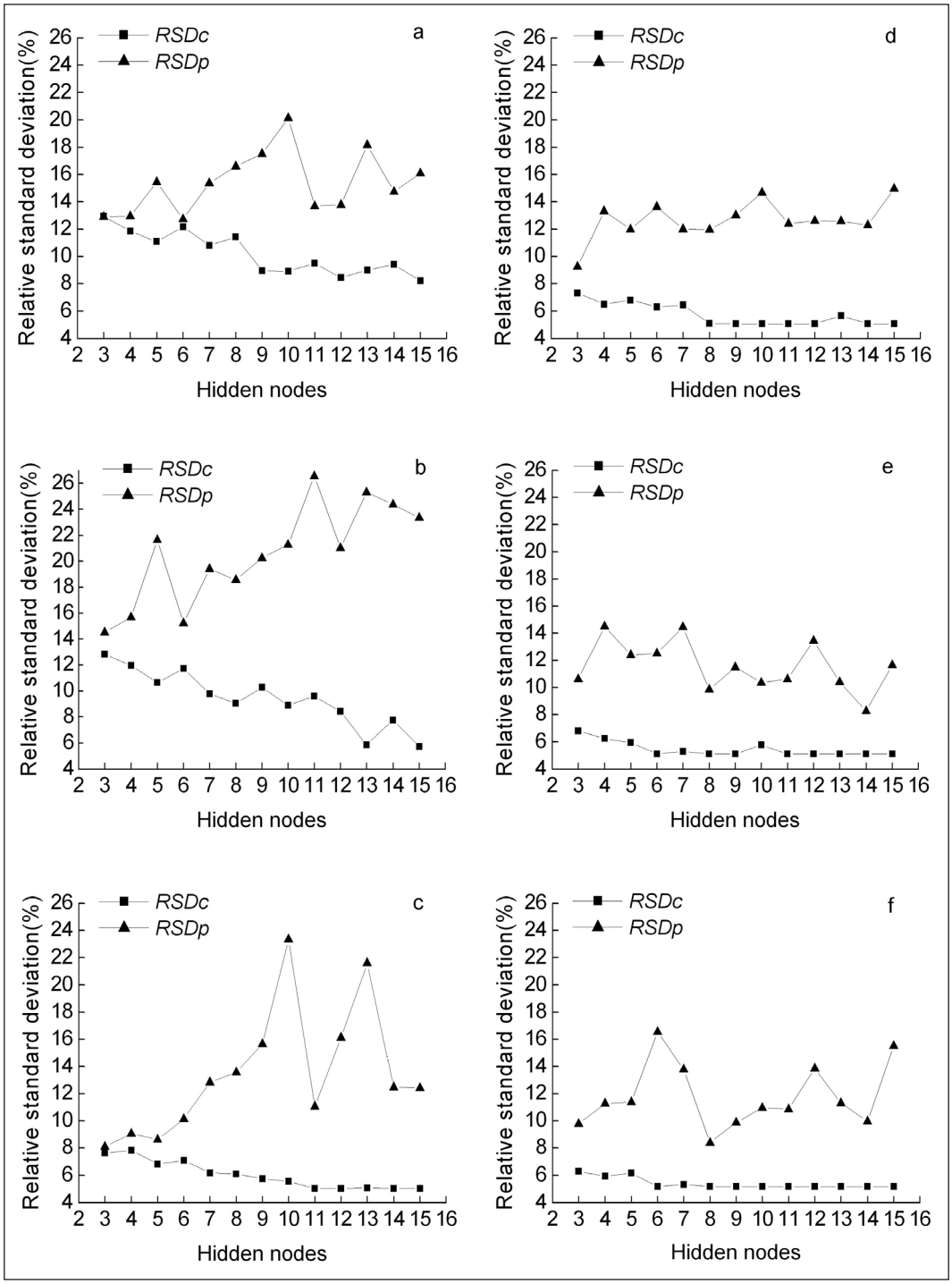


Fig. 1 - *RSD* under different network structures:(a) three input nodes; (b) four input nodes; (c) five input nodes; (d) six input nodes; (e) seven input nodes; (f) eight input nodes.

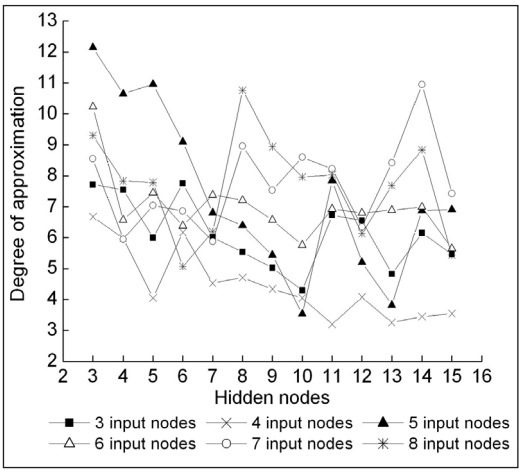


Fig. 2 - Degree of approximation under different network structures.

nodes were five and three, respectively, the network had the largest degree of approximation, resulting in the most stable network structure (5, 3, 1).

Selection of training function

In the MATLAB Neural Network Toolbox (DEMUTH and BEALE, 1998), there is a large number of network training functions which update weight and bias values through various algorithms. The selection of training methods depends on the application due to the difference in the nature of the nonlinearities. In this part, eight types of training techniques were tested and compared with a view to establishing models of a high D_a for the determination of SSC in apples. The functions and relevant properties are listed in Table 3. It can be concluded that the performance of different networks was affected appreciably with respective training function.

However, although the latter four training functions were taken on the corresponding ANN models with larger r and smaller RSD_C than other methods, their RSD_P were exceeded or approached 10%, suggesting that the models did not have good predictive ability. In Table 3, the Traingdm function producing the highest D_a (11.568) proved to be the most efficacious training tool for analyzing networks in this experiment.

Determination of learning coefficient and momentum

Prior to the start of training, the learning coefficient was initialized with a random value. However, the learning coefficient affects the training speed of the

Table 3 - The results of training functions.

Training function	Methods of weight and bias update	r[a]	RSD_C[b](%)	RSD_P[c](%)	D_a[d]
Traingd	General gradient descent	0.879	7.56	8.47	11.466
Traingdm	Gradient descent with momentum	0.877	7.59	8.41	11.568
Traingda	Gradient descent with adaptive learning rate	0.866	7.90	8.98	10.781
Traingdx	Gradient descent with momentum and an adaptive learning rate	0.877	7.59	9.31	10.214
Trainrp	The resilient back-propagation algorithm	0.921	6.15	10.14	8.886
Trainlm	Levenberg-Marquardt optimization	0.895	7.04	9.41	9.929
Trainbfg	The BFGS quasi-Newton method	0.906	6.69	9.19	10.112
Traincgb	The conjugate gradient back-propagation with Powell-Beale restarts	0.897	6.99	11.36	7.945

[a] correlation coefficient
[b] relative standard deviation of calibration
[c] relative standard deviation of prediction
[d] degree of approximation.

network training. Generally, too high of a learning rate leads to local minimum during the descent down the error surface, and too low of a value results in slow convergence. The effect of the coefficient on the network did not change (Fig. 3). The *r* changed between 0.86 and 0.89, while the RSD_C was almost stable, and the RSD_P changed a little. But from the D_a shown in Fig. 3 (c), it was easy to make a decision that the network had the highest D_a at the coefficient of 0.20.

Before training, the momentum was set to a random value. In this work, the effect of momentum was explored during network training. Too large a momentum leads to oscillation and training divergence, whereas too small a momentum takes a long training time. As shown in Fig. 4, the difference of the RSD_C was small and the RSD_P was stable except for 0.35 and 0.75 of momentum. The values of *r* varied between 0.865 and 0.895.

The D_a could present the error of the network and it was easy to make a decision on the basis of D_a. The effect of D_a on the network was regular with the change of momentum (Fig. 4 (c)). The network had the highest D_a at the momentum of 0.8.

Determination of number of iterations

The number of iterations was also an adjustable parameter. By increasing the number of iterations, the training accuracy was improved. However, the prediction errors also increased as shown in Fig. 5. When the training cycles exceeded 25000, the D_a was reduced greatly. This result demonstrated that there was over-fitting in the network. It was easy to choose the suitable number of iterations from Fig. 5 (c). The network reached a maximal D_a at 25000 iterations; 25000 was the optimal number of iterations in this procedure.

Finally, the specifications for the networks are listed in Table 4. All the op-

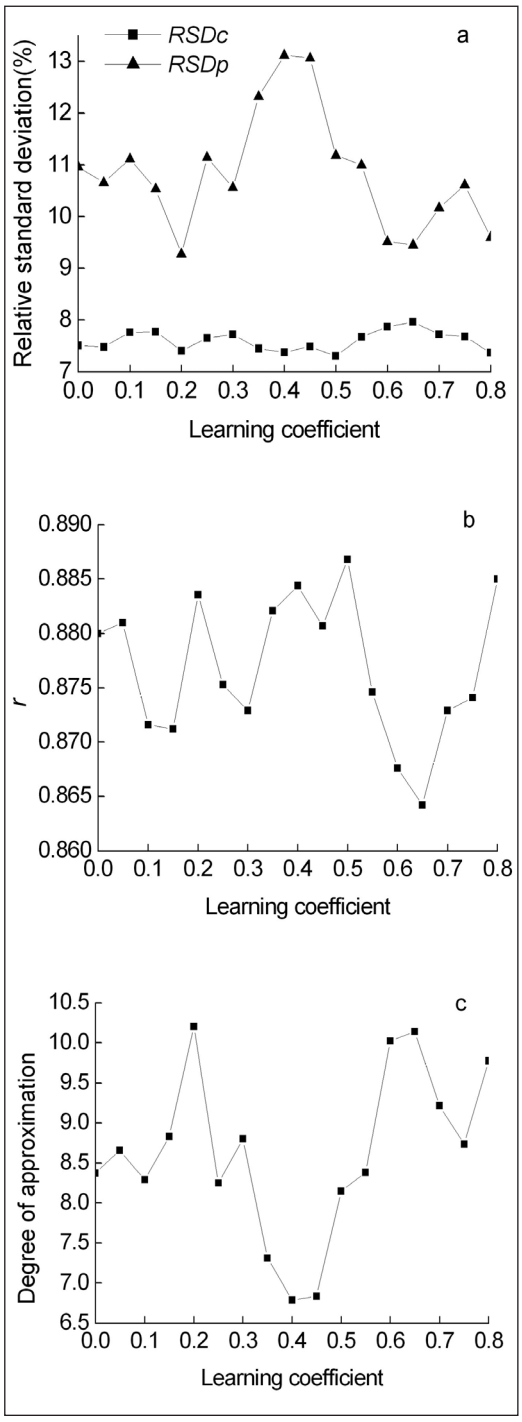


Fig. 3 - Effect of learning coefficient: (a) RSD_C and RSD_P; (b) *r* of calibration set; (c) Degree of approximation.

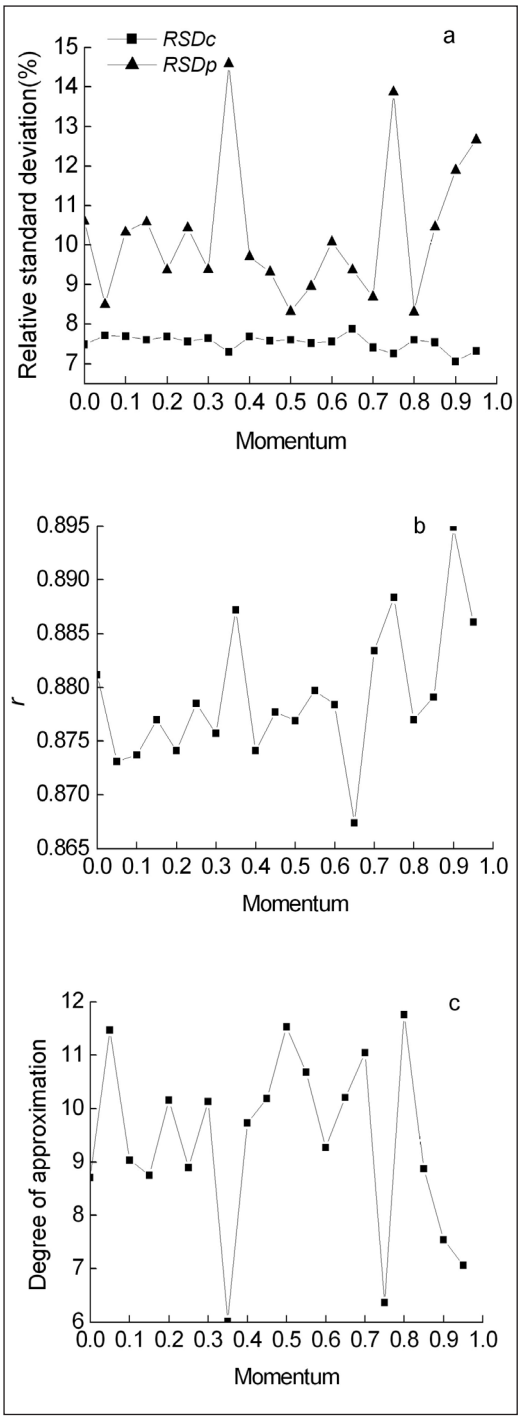


Fig. 4 - Effect of momentum: (a) RSD_C and RSD_P; (b) r of calibration set; (c) Degree of approximation.

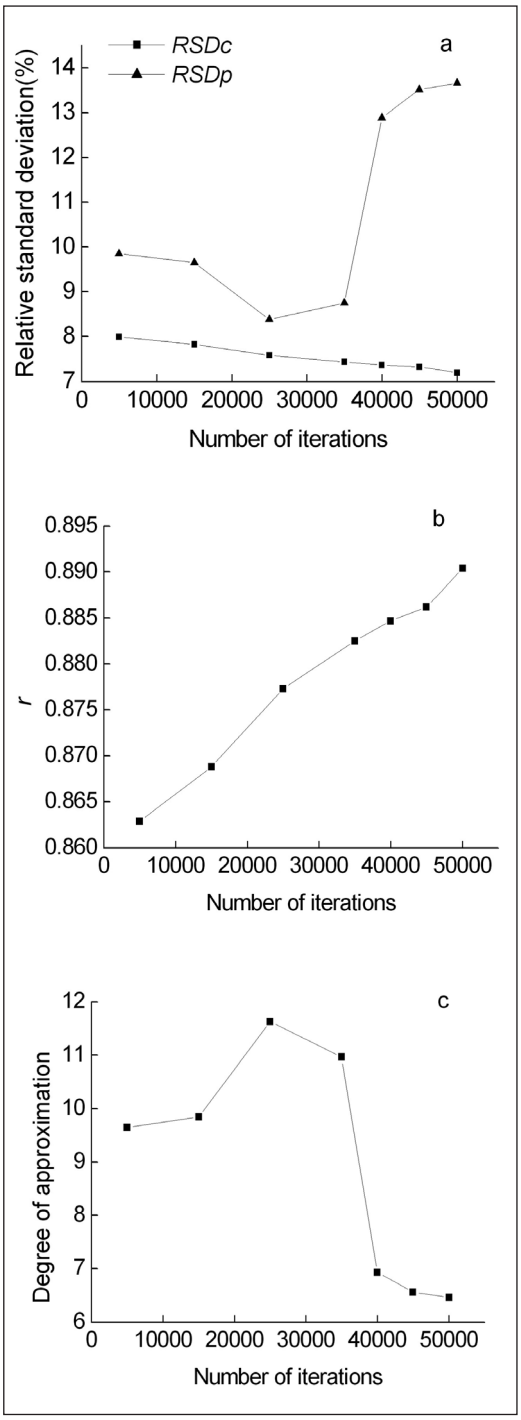


Fig. 5 - Effect of number of iterations: (a) RSD_C and RSD_P; (b) r of calibration set; (c) Degree of approximation.

timal parameters and structure were found to be optimal for fast learning with low prediction errors. According to the whole program of parameters and structure optimization, selection of the number of input and hidden nodes played an important role in ANN training and the prediction capability. In contrast, the learning coefficient, momentum and the number of iterations had only a small effect on the network, thus they were not the primary factors when the network was established.

Effect of weight initializations

In the network model, the training error was also influenced by initial weights. At the start of a training run, the weights were initialized with random values by initialization function. Based on the optimal network structure and selected parameters, attempts were made to obtain appropriate connection weights with improved prediction capabilities by using the initialization of weights. In this project, the Nguyen-Widrow initialization method (NGUYEN and WIDROW, 1990) was used and weights were reinitiated nine times to retrain the neural networks. Fig. 6 show the results with varied initial weights. The optimization of network weights was obtained the fifth time with the largest D_a (11.625).

After the ANN training was completed

Table 4 - Optimal parameters and structure of BP-ANN.

Input layer transfer function	Linear
Hidden layer transfer function	Hyperbolic tangent
Output layer transfer function	Linear
Training function	Traingdm
Input nodes	5
Hidden nodes	3
Output nodes	1
Learning coefficient	0.2
Momentum	0.8
Number of iterations	25000
Mean square error (MSE)	0.1

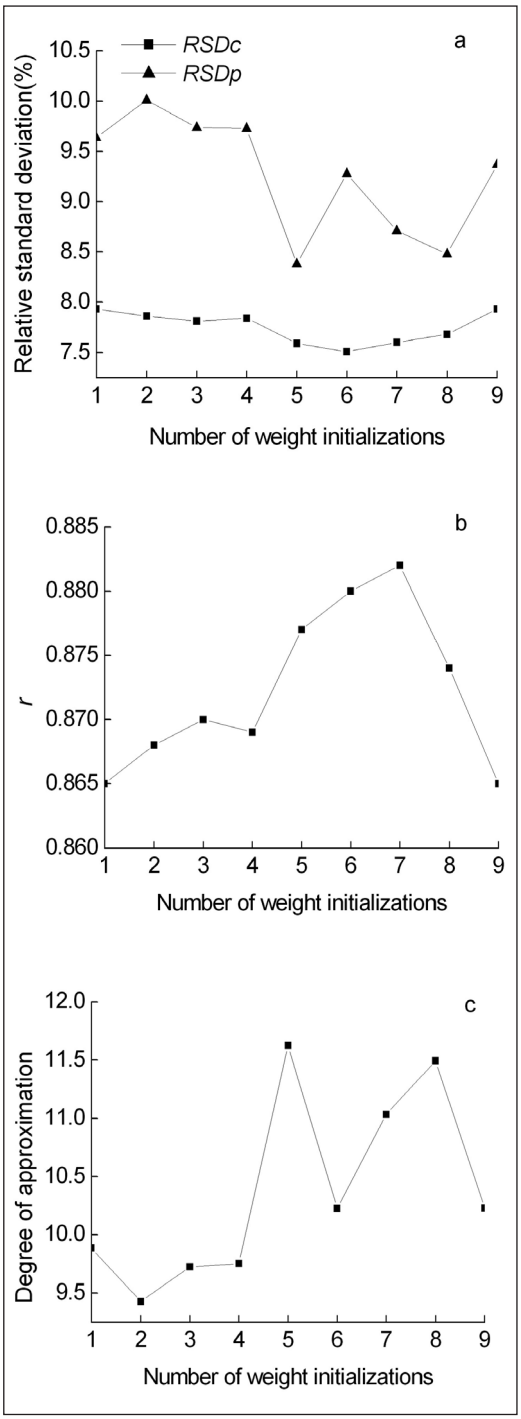


Fig. 6 - Effect of initial weights: (a) RSD_c and RSD_p; (b) r of calibration set; (c) Degree of approximation.

in the fifth model, all of the weights within the ANN were fixed. Both the weights from the input to hidden and from the hidden layer to the output were calculated as follows:

$$w1 = \begin{bmatrix} 0.0005 & -0.0098 & 0.0920 & 0.7659 & 3.1815 \\ 0.0266 & 0.0062 & 1.2776 & -2.4788 & 0.8189 \\ 0.0665 & -0.2977 & 0.7484 & -1.0082 & 3.1290 \end{bmatrix}$$

$$b1 = \begin{bmatrix} -0.5453 \\ -0.1808 \\ -2.5994 \end{bmatrix}$$

$$w2 = \begin{bmatrix} 2.2974 & 0.7909 & -0.9550 \end{bmatrix}$$

$$b2 = \begin{bmatrix} 0.1606 \end{bmatrix}$$

In the following, a formal description of this ANN function is presented

$$y = purelin(w2 \times \tan sig(w1 \times z + b1) + b2) \quad (8)$$

where *w1* is the weight connecting the input with the hidden layer neuron; *b1* is a bias for each neuron in the hidden layer; *w2* is the weight connecting the neuron in the hidden layer to the output layer; *b2* is a bias for the output; tan *sig* (hyperbolic tangent function) is the transfer function in the hidden layer; *purelin* (linear function) is the transfer function in the output; *z* is the principal component scores on spectral data of samples; and *y* is the SSC predicted by the model. Thus, the prediction will be performed by equation (8). Under the optimal parameters, initial weights and structure of BP-ANN, the best model was established (r=0.877, RSD_C=7.59%, RSD_P=8.38%).

PLSR models based on spectra using different pre-treatments

The raw spectra of training samples are shown in Fig. 7 (a). The observable absorbance peaks between 1420-1490nm corresponded to the first O-H stretching overtone, by reason of overlapping of the specific water absorption band (OSBORNE and FEARN, 1988). However, the absorbance bands of SSC were not observed directly in the spectra. The position of bands can be shifted by temperature changes or hydrogen bonding interactions with sample components. In this work, three spectral pre-treatments were attempted to decrease the shiftiness of spectra. Multiple scatter correction (MSC) reduced dispersions which resulted in multiplicative deviation. It was clear to see that the profile of the spectra was merged after MSC (Fig. 7 (b)), suggesting that light scattering was produced by the apples. To reduce baseline drift and overlap, first and second derivatives were attempted (Fig. 7(c; d)). However, after derivatives, the signal of the spectra showed a low intensity due to the low concentration of chemical material.

The statistical results of PLSR following different pre-treating are reported in Table 5. It is obvious that the preprocessing of derivative was unable to improve the capability of predication. Although enhancing the baseline correction and removing constant background signals, derivative increased the noise and reduced the signal-to-noise ratio, resulting in a higher RSD_P. This was a disadvantage of the derivative with a view to useful low-information parts of the signal. MSC was a valuable tool for eliminating scattering that was changed in the optical path-length and possibly in the sensitivity of the detector and the amplifier. The results showed that MSC had light improved the predication attributes (RSD_P=8.76%). According to the results, non-linearity in the signal-analyte concentration relation did not arise from scattering and overlapping. Therefore, the pre-treatments in this work were not absolutely perfect to robust prediction performance. Nevertheless, compared with the results of PLSR, the improvement of the quality of the training

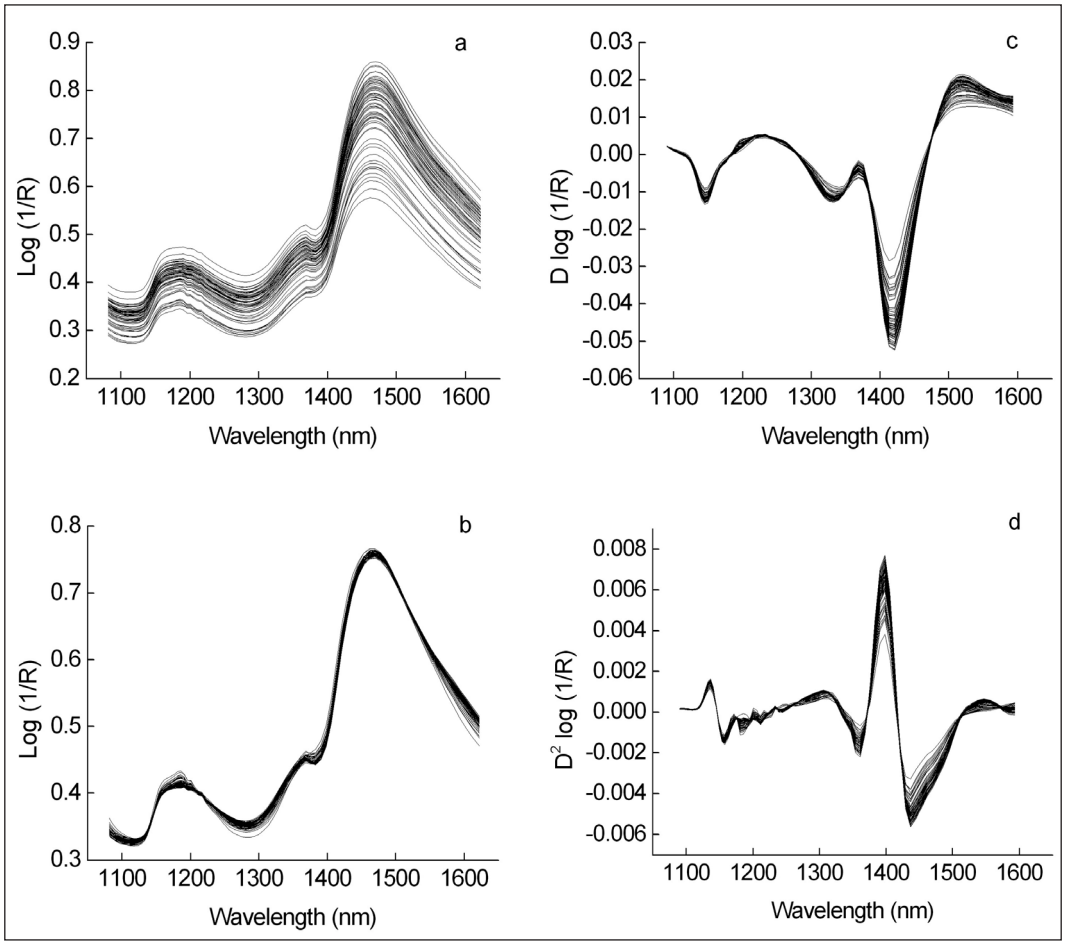


Fig. 7 - The spectra of calibration samples by different pre-treatments: (a) the original spectra; (b) MSC; (c) First derivative; (d) Second derivative.

Table 5 - Implementation of various spectral pre-treatments before PLSR calibration.

Pre-processing	Number of LV[d]	*r* [e]	RSD_c [f](%)	RSD_p [g](%)
none	5	0.869	7.82	8.89
MSC[a]	5	0.867	7.86	8.76
D log (1/R)[b]	7	0.885	7.47	9.06
D^2 log (1/R)[c]	5	0.870	7.81	11.33

[a] multiple scatter correction
[b] first derivative
[c] second derivative
[d] latent variables
[e] correlation coefficient
[f] relative standard deviation of calibration
[g] relative standard deviation of prediction.

model was brought with *r* of 8.77, RSD_c of 7.59% and RSD_p of 8.38% by the optimal ANN function. The source of non-linearity was related to the chemical nature of the target parameter (SSC). The non-linearity in the absorption band of interest was intrinsic and cannot be corrected by spectral pretreatment.

CONCLUSIONS

The spectral pre-treatments did not absolutely resolve the nonlinearity, suggesting that the spectral data of apples had intrinsic nonlinearity. In contrast, the neural network performed slightly better capability (*r*=0.877, RSD_C=7.59%, RSD_p=8.38%) than PLSR (*r=0.867*, RSD_C*=7.86%*, RSD_p*=8.76%*) on determination of SSC in internal apples. During ANN training, the network structure (5, 3, 1) was mainly important to establish the mathematically corrected model, and the parameters did not have significant effects on the network. Finally, it can be concluded that AOTF-NIR spectroscopy and BP-ANN with PCA can be properly employed for rapid and nondestructive determination of internal apple quality.

ACKNOWLEDGEMENTS

This study was supported by the Key Technologies Program of the Ministry of Science and Technology, People's Republic of China. (Project No.:2001BA501A16B).

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Revised paper received May 14, 2007 Accepted September 4, 2007

PAPER

ENZYMATIC SYNTHESIS OF STRUCTURED 1,2-DIACYL-*sn*-GLYCERO-3-PHOSPHOCHOLINES FROM GLYCEROL-*sn*-3-PHOSPHOCHOLINE

SINTESI ENZIMATICA DI 1,2-DIACIL-*sn*-GLICERO-3-FOSFATIDILCOLINE STRUTTURATE DA GLICERO-*sn*-3-FOSFOCOLINA

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ABSTRACT

The synthesis of structured 1,2-diacyl-*sn*-glycero-3-phosphocholines (*sn*-1,2-PC) from glycerol-*sn*-3-phosphocholine (GPC) was studied by means of enzymatic acylation reactions of the two free *sn*- positions of the glycerol backbone. Already after 22 h high conversions (absolute yields = 100%) of GPC to *sn*-2-lysophosphatidylcholines (*sn*-2-LPC) were achieved using Novozym 435,

RIASSUNTO

Il presente lavoro tratta la sintesi di fosfatidilcoline (*sn*-1,2-PC) strutturate a partire dalla glicero-*sn*-3-fosfocolina (GPC) mediante reazioni di acilazione enzimatica delle due *sn*- posizioni dello scheletro glicerolico. Già dopo 22 h sono state ottenute alte conversioni (resa assoluta = 100%) del GPC a *sn*-2-lisofosfatidilcoline (*sn*-2-LPC) utilizzando il Novozima 435, lipasi da *Can-*

- Key words: 1,2-diacyl-*sn*-glycero-3-phosphocholine, *sn*-glycerol-3-phosphocholine, Novozym 435, Phospholipase A_2 -

a lipase from *Candida antarctica*, selective for the *sn*-1-position of GPC. In the second step the acylation of the *sn*-2-position of the *sn*-2-LPC was catalysed by phospholipase A_2 from hog pancreas and better acylation yields (40% absolute yield, corresponding to a relative yield of 70%, when the reactions were carried out at controlled water activity) were obtained if compared to analogous reactions reported in the literature. A chemical procedure to obtain structured *sn*-1,2-PC from *sn*-2-LPC was also used and better results (65% absolute yield, corresponding to a relative yield of 95%) were obtained in comparison with the enzymatic method.

dida antarctica, selettiva per la posizione *sn*-1- del GPC. Nel secondo passaggio, l'acilazione della posizione *sn*-2- delle *sn*-2-LPC è stata catalizzata dalla fosfolipasi A_2 suina, ottenendo migliori rese di acilazione (resa assoluta del 40%, corrispondente ad una resa relativa del 70%, quando le reazioni sono state condotte in condizioni di attività dell'acqua controllata) rispetto a simili reazioni riportate in letteratura. È stata inoltre effettuata una procedura chimica per ottenere *sn*-1,2-PC strutturati a partire da *sn*-2-LPC e sono stati ottenuti migliori risultati (resa assoluta del 65%, corrispondente ad una resa relativa del 95%) rispetto a quelli della via enzimatica.

INTRODUCTION

An increasing demand for structurally defined phospholipids (PL) in the pharmaceutical, food, cosmetic and other fields has stimulated the development of different synthesis procedures (ADLERCREUTZ *et al.*, 2002).

Structured PL can be enzymatically manufactured by incorporating fatty acids (FA) with specific biological functions into natural PL. The modification of PL has been carried out by phospholipase or lipase-catalyzed transesterifications and/or acidolysis reactions (PERNAS *et al.*, 1990; PENG *et al.*, 2002).

The *sn*-glycerol-3-phosphocholine (GPC) has been used as a starting compound for the synthesis of structured lysophosphatidylcholines (LPC). Lipases (EC 3.1.1.3; triacylglycerol acyl hydrolases) have been used extensively as a valuable alternative to chemical methods. MAZUR *et al.* (1991) reported the esterification of GPC with FA anhydrides (71% conversion), catalysed by a 1,3-specific lipase. KIM and KIM (2000) used free FA as acyl donors (88% conversion). VIRTO and ADLERCREUTZ (2000) carried out an acylation reaction of GPC with FA vinyl esters as acyl donors (-LPC+PC-mol% >95).

In the most common procedures for PL synthesis, *sn*-2-LPC were used as starting material for enzymatic or chemical esterification reactions (NICHOLAS *et al.*, 1983; EGGER *et al.*, 1997). The chemical reactions are often simple, inexpensive, but less attractive for medical and biological applications (DE GREYT *et al.*, 1998). Numerous advantages can be obtained by using enzymes to catalyse the reactions for lipid modifications. Briefly, the enzymatic reactions are characterized by selectivity, mild reaction conditions and good acceptability by consumers (GUNSTONE, 1999; IWASAKI and YAMANE, 2000).

Several studies have been reported on the synthetic activity of phospholipase A_2 (PLA_2) in different organic media to modify the *sn*-2- position of PL. NA *et al.* (1990) reported the synthetic reaction in a microemulsion medium from which a low PC yield (about 6%) was obtained. MINGARRO *et al.* (1994) showed that the

highest activity of PLA_2 was obtained using aromatic hydrocarbons (toluene, benzene); it was observed that the activity generally decreased as polarity increased. On the other hand, PARK *et al.* (2000) carried out the PLA_2-catalysed interesterification of PC with ethyl esters of eicosapentaenoic acid in various organic solvents; they found that the highest incorporation yield was obtained in toluene (about 15%). HOSOKAWA *et al.* (1995) carried out the esterification of LPC with eicosapentaenoic acid or docosahexaenoic acid in glycerol and studied the effect of different water mimics; the highest yield (about 60%), calculated as the ratio between synthesized PC (mg) and substrate LPC (mg), was obtained in the presence of dimethylformamide. EGGER *et al.* (1997) synthesized PC from *sn*-2-LPC and FA; they studied the dependence of the reaction rate on chain length and the degree of FA unsaturation using PLA_2 in toluene. To minimize hydrolysis during synthesis, a water activity (a_W) gradient technique has been suggested (GUO *et al.*, 2005).

In this work, the enzymatic acylation of GPC, obtained from natural PC by enzymatic deacylation reported in a previous paper (BLASI *et al.*, 2006), was carried out with Novozym 435, lipase from *Candida antarctica* which is very selective for the *sn*-1-position, to esterify capric or lauric acid, until most of the substrate was consumed. The successive step was relative to the enzymatic incorporation of oleic acid in the *sn*-2-position of *sn*-2-LPC to obtain structured *sn*-1,2-PC, using PLA_2 from hog pancreas in glycerol; chemical acylation of the *sn*-2- position of *sn*-2-LPC was also carried out.

MATERIALS

sn-2-LPC and *sn*-1,2-PC from egg yolk, methanol (MeOH), chloroform ($CHCl_3$) and anhydrous *t*-butanol (analytical grade, ≥99.0%), were purchased from Sigma-Aldrich (St. Louis, MO). *N,N'*-dicyclohexylcarbodiimide (DCCD), 4-dimethylaminopyridine (DMAP), anhydrous oleic acid, vinyl laurate, vinyl caprate, sodium bis-2-ethylhexyl-sulfosuccinate (AOT) and anhydrous glycerol (≥99.0%) were purchased from Fluka (Buchs, Switzerland). *N,N*-dimethylformamide, potassium acetate (CH_3COOK), calcium chloride ($CaCl_2$), ethanol (EtOH) (analytical grade) and MeOH (HPLC grade) (≥ 99.0%) were supplied by Carlo Erba Reagents (Milan, Italy). Water (H_2O) (HPLC grade) was from J.T. Baker (Deventer, Holland).

Lipase B (EC 3.1.1.3; triacylglycerol acyl hydrolases) from *C. antarctica* (Novozym 435), immobilized on macroporous acrylic resin, recombinant, expressed in *Aspergillus oryzae*, ≥10,000 U/g, was acquired from Sigma (St. Louis, MO) and phospholipase A_2 from hog pancreas (EC 3.1.1.4; phosphatidylcholine 2-acylhydrolases), 561 U/mg, was obtained from Fluka (Buchs, Switzerland).

Lipase from *Mucor miehei* (EC 3.1.1.1; carboxylic-ester hydrolase; Lipozyme IM), immobilized on anionic resin, 42 U/g, was from Novo Nordisk A/S (Bagsvaerd, Denmark).

Molecular sieve beads (type 4A, diameter 1.7-2.4 mm) were supplied by Fluka (Buchs, Switzerland); silica gel plates SIL G-25 (20 x 20 cm) were from Macherey-Nagel (Düren, Germany).

Silica gel plates (SIL G-25, 20×20 cm, 0.25 mm) were obtained from Macherey-Nagel (Düren, Germany).

METHODS

The two steps for the synthesis of *sn*-1,2-PC, starting from GPC are represented in the following scheme:



1. Enzymatic synthesis of *sn*-2-LPC

GPC was prepared from *sn*-1,2-PC by deacylation in a microemulsion system as described by BLASI *et al.* (2006). Briefly, *sn*-1,2-PC (about 5 mg) were dissolved in 0.75 mL of isooctane, then AOT (7.5 mg) and 0.15 mL of borate buffer (0.1 M; $CaCl_2$ 1 mM; pH 7.5) were added; Lipozyme IM (5 mg) and phospholipase A_2 (1 mg) were then added to the reaction mixture. The reaction was carried out under magnetic stirring at 35°C for 48 h. After this time, the reaction mixture was washed initially with 2 mL of $CHCl_3$:MeOH (2:1 v/v), 2 mL of $CHCl_3$:MeOH (1:1 v/v) and after with MeOH (1.5 mL); the extracts collected were filtered, concentrated and applied to a silica gel plate; thin-layer chromatography (TLC) was performed using $CHCl_3$:MeOH:H_2O (30:50:20 v/v/v) as developing mixture.

The *sn*-2-LPC were obtained according to the following procedure: 0.025 mmol GPC (obtained from about 20 mg of *sn*-1,2-PC), 1 mmol FA (capric or lauric acid vinyl esters) vinyl ester and 5 mg Novozym 435, without *t*-butanol or with 50% (v/v) *t*-butanol/substrate solution and with different quantities of water, were incubated under magnetic stirring (100 rpm); the reactions were carried out at 30°C and 45°C, for 22 and 100 h.

The enzymatic reaction was stopped by adding 1 mL of EtOH:H_2O (95:5 v/v), the mixture was then dried under nitrogen stream; the residue was washed with $CHCl_3$:MeOH (2:1 v/v) and filtered. An aliquot (20 μL) was analyzed by high performance liquid chromatography equipped with evaporative light scattering detector (HPLC-ELSD), as reported in Section 3.

The absolute yield (%) of *sn*-2-LPC was calculated as follows:

$$\textit{absolute yield (\%) of sn-2-LPC} = \frac{\textit{synthesized sn-2-LPC}}{\textit{theoretical sn-2-LPC}} \times 100$$

where: *synthesized sn-2-LPC* were the mg of *sn*-2-LPC calculated using the HPLC peak area and a standard curve, obtained as reported in Section 3. and *theoretical sn-2-LPC* corresponded to the mg of *sn*-2-LPC formed after the total conversion of GPC.

The *sn*-2-LPC obtained were isolated by TLC on silica gel plates using $CHCl_3$:MeOH:H_2O (50:40:10 v/v/v) as developing mixture.

2-a. Enzymatic acylation of *sn*-2-LPC to synthesize *sn*-1,2-PC

Enzymatic acylation of *sn*-2-LPC was carried out by modifying the experimental conditions used by HOSOKAWA *et al.* (1995). Ten mg of *sn*-2-LPC (obtained and isolated as reported in Section 1.), 23 μL anhydrous oleic acid and 10 mg of PLA_2 were added to 500 mg of anhydrous glycerol. The enzymatic reactions were carried out under the following conditions:

a) without dimethylformamide;

b) with anhydrous dimethylformamide (0.1 mL) and molecular sieves (about 35 mg);

c) with anhydrous dimethylformamide (0.1 mL), in a super-saturated CH_3COOK salt solution (a_w = 0.22), in order to control the a_W during the reaction. The a_W of the reaction medium and of the enzyme preparation were equilibrated overnight, in a closed container in a super-saturated CH_3COOK solution, at 25°C. The reaction was initiated by adding the enzyme preparation to the reaction medium and carried out in the salt container.

The acylation reaction was carried out under magnetic stirring for 48 h at 25°C and then stopped by adding 5 mL of $CHCl_3$:MeOH:H_2O (10:5:3 v/v/v). The chloroformic phase was recovered; the upper phase was washed with $CHCl_3$:MeOH:H_2O (86:14:1 v/v/v) and the lower phase was added to the preceding one and then dried under nitro-

gen stream. The residue was dissolved in $CHCl_3$:MeOH (2:1 v/v); an aliquot (20 μL) was filtered and analysed by HPLC-ELSD, as reported in Section 3.

The absolute yield (%) of *sn*-1,2-PC was calculated as follows:

$$\textit{absolute yield (\%) of sn-1,2-PC} = \frac{\textit{synthesized sn-1,2-PC}}{\textit{theoretical sn-1,2-PC}} \times 100$$

where: *synthesized sn-1,2-PC* were the mg of *sn*-1,2-PC calculated using HPLC peak area and a standard curve, obtained as reported in Section 3 and *theoretical sn-1,2-PC* corresponded to the mg of *sn*-1,2-PC formed after total conversion of *sn*-2-LPC.

The *sn*-1,2-PC yield was also calculated as *sn*-1,2-PC relative yield (%), as reported by HOSOKAWA *et al.* (1995):

$$\textit{relative yield (\%) of sn-1,2-PC} = \frac{\textit{synthesized sn-1,2-PC}}{\textit{substrate sn-2-LPC}} \times 100$$

where: *substrate sn-2-LPC* were the mg of *sn*-2-LPC used for the reaction of PC synthesis.

The *sn*-1,2-PC obtained were isolated by TLC on silica gel plates using $CHCl_3$:MeOH:H_2O (50:40:10 v/v/v) as developing mixture.

2-b. Chemical acylation of *sn*-2-LPC to synthesize *sn*-1,2-PC

A solution of *sn*-2-LPC (1.0 mmol, obtained and isolated as reported in Section 1), anhydrous oleic acid (2.0 mmol), DCCD (4.0 mmol) and DMAP (4.0 mmol) in 0.5 mL ethanol-free chloroform was stirred at room temperature overnight (ROSU *et al.*, 1999). The mixture was dried under nitrogen stream, then the residue was dissolved in $CHCl_3$:MeOH (2:1 v/v) and an aliquot (20 μL) was filtered and analysed by HPLC-ELSD, as reported in Section 3.

The *sn*-1,2-PC obtained were isolated as reported in Section 2a.

3. HPLC-ELSD analysis

Apparatus. The HPLC analyses were carried out using a gradient pump Model 305 (Gilson, Middletown, WI, USA), a Lichrosorb Si-60 (3 μm, 250 x 4.0 mm i.d., Merck, Darmstad, Germany) column, a ELSD (Sedex 55, S.E.D.E.RE., France), operating at 35°C at nitrogen pressure of 2.3 atm. The chromatograms and data were acquired and processed using the Class-VP software (Shimadzu, Kyto, Japan).

Chromatographic conditions. The filtered samples were analysed by gradient elution: from 100% MeOH to mixture 80% MeOH and 20% H_2O in 15 min; this composition was held for 10 min, then the column was reconditioned with 100% MeOH. The mobile phase flow was 1 mL/min.

Ranges of retention times were: 6-7 min for *sn*-1,2-PC, 9.5-10.5 min for *sn*-2-LPC and 15-16 min for GPC.

The quantification of the reaction products, produced by enzymatic or chemical procedure, was carried out using calibration curves, built analysing known quantities of *sn*-2-LPC and *sn*-1,2-PC standard solutions (0.25, 0.5, 1.0, 1.5, 2.0 mg/mL); the detector response was linear in this range (correlation coefficients were 0.9969 and 0.9897 for *sn*-2-LPC and *sn*-1,2-PC, respectively).

RESULTS AND DISCUSSION

1. Enzymatic synthesis of *sn*-2-LPC

The *sn*-2-LPC and *sn*-1,2-PC standards were analysed by HPLC and the results were used to build the calibration curves necessary to carry out the quantitative analysis of LPC and PC produced by enzymatic or chemical procedures.

The esterification of GPC to obtain *sn*-

2-LPC (Section 1) was carried out in a solvent-free system or in the presence of 50% (v/v) *t*-butanol, with "activated" FA in the form of vinyl esters. The use of these compounds in esterification reactions is advantageous because the vinyl alcohol released during the reaction tautomerizes to acetaldehyde, which cannot act as substrate of the enzyme used. The released acetaldehyde deactivates many enzymes, but not the lipase from *C. antarctica* (BERGER and FABER, 1991). Novozym 435 (lipase from *C. antarctica*) was very selective for the *sn*-1-position; in fact the formation of PC was not detected during the first 22 h of the reaction, the time required for substrate consumption.

The yield of esterification of GPC with capric acid vinyl ester to *sn*-2-LPC was 60% when the reaction was carried out for 22 h at 45°C in water-free medium but it decreased to 42%, with formation of *sn*-1,2-PC (7%) after 100 h. The HPLC profile of these acylation reactions never showed the presence of GPC; this occurrence could be explained by GPC adsorption on the macroporous resin of the enzyme particles. In fact, control reactions were carried out in the same conditions reported in Section 1. without FA vinyl esters and an incomplete recovery of GPC was observed.

The PC detected when the enzymatic reaction was carried out for 100 h was derived from the acylation of the *sn*-1-LPC, which formed when the acyl group of the *sn*-2-LPC spontaneously migrated from the *sn*-1- to the *sn*-2- position (BLASI *et al.*, 2006).

To increase the acylation yields of the GPC *sn*-1- position, the water level should be kept as low as possible, but enough to allow the synthetic enzyme activity; however, in the presence of 0.1 mmol of water, the best results were achieved in the presence of 50% (v/v) *t*-butanol, a water-miscible solvent with low hydrophobicity, that improved the mixing of the substrates (Fig. 1). Successively the reactions were always carried out in the presence of 50% (v/v) *t*-butanol.

The effects of the various initial water contents and of acyl chain length are reported in Fig. 2 a and b; when the reaction was carried out with vinyl laurate at 45°C, the yield of *sn*-2-LPC increased up to 55% and 85% by adding 0.5 mmol of water and 0.1 mmol of water, respectively. Yields of 60, 80 and 65% were ob-

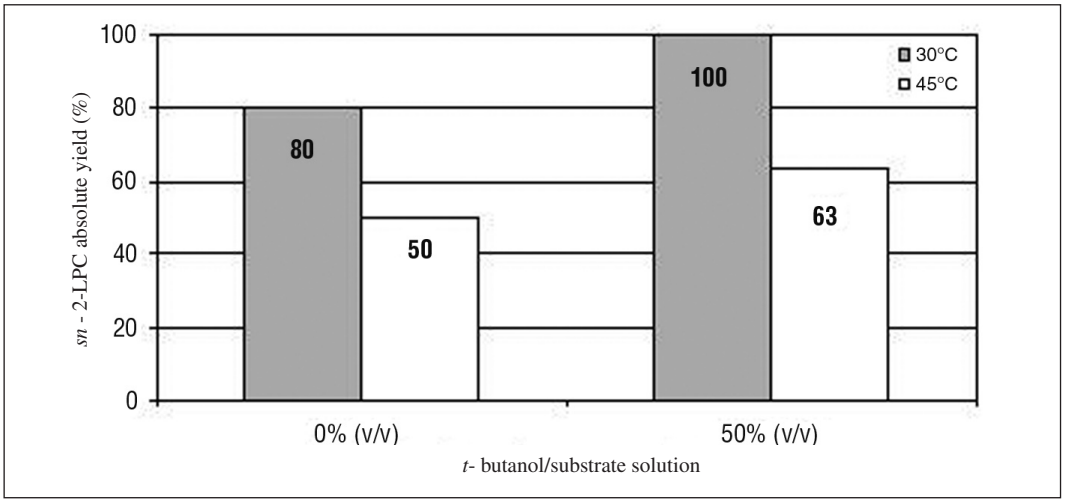


Fig. 1 - Absolute yields (%) of *sn*-2-LPC, obtained by GPC esterification with vinyl caprate, 22 h, 0.1 mmol water; the relative standard deviation values were never higher than 5.2% (n=3).

tained when the reactions were carried out at 30°C with the addition of 0.0, 0.1 and 0.5 mmol of water, respectively (Fig. 2a). It was observed that the LPC yield depended on the acyl chain length of the vinyl ester; higher yields were observed with vinyl laurate at 45°C (Fig. 2a) and with vinyl caprate at 30°C (Fig. 2b).

The highest yield (100%) was obtained with vinyl caprate at 30°C for 22 h with 0.1 mmol of water and 50% (v/v) *t*-butanol.

2a. Enzymatic acylation of *sn*-2-LPC to synthesize *sn*-1,2-PC

As the second step of the study, the conditions of an enzymatic procedure for the acylation of the *sn*-2- position of *sn*-2-LPC, based on the use of PLA_2 from hog pancreas (Section 2a) were studied. The quantity of water in an organic reaction medium influences the yields and the enzymatic kinetics. Under completely water-free conditions, no enzyme ac-

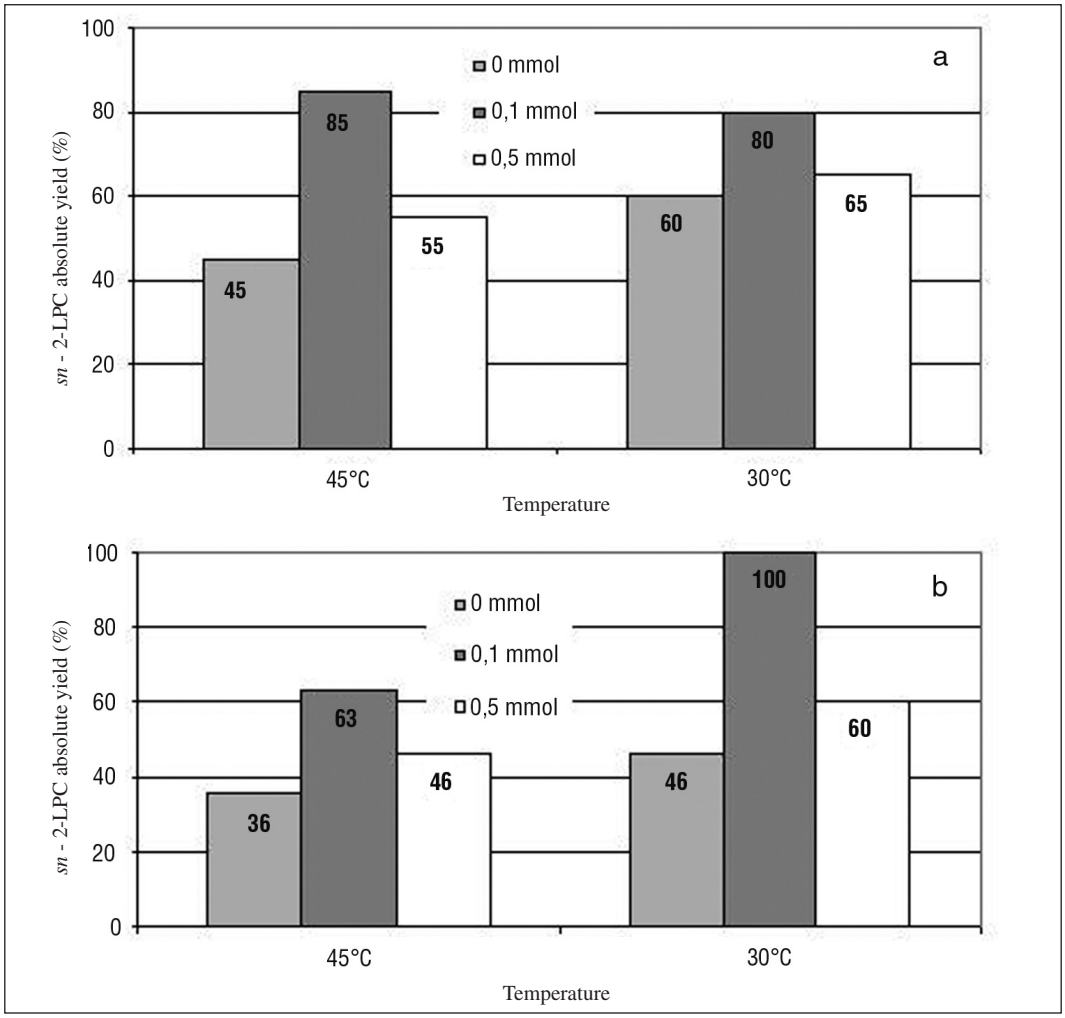


Fig. 2 - Absolute yields (%) of *sn*-2-LPC, obtained by GPC esterification with vinyl laurate (a) and vinyl caprate (b), 22 h at 45°C and 30°C, at different water contents, in the presence of *t*-butanol; the relative standard deviation values were never higher than 4.0% (n=3).

tivity is expressed (EGGER *et al.*, 1997); thus, some controlled water quantities must be added, because the a_w determines the degree of hydration of the enzyme and thus the catalytic activity.

In the present work the enzymatic reactions were carried out with or without dimethylformamide. This non-aqueous solvent is a valuable water mimic medium, capable of forming multiple hydrogen bonds. It can therefore be used as a water substitute and its use could be favourable in esterification reactions. In fact, small amounts of dimethylformamide enhanced FA incorporation and higher yields were obtained. As shown in Fig. 3, the absolute yield reached 30% (relative yield = 47%) with the use of dimethylformamide and with molecular sieves, but the absolute yield increased to 40% (relative yield = 70%) when the reaction was carried out in the presence of dimethylformamide, over a saturated CH_3COOK solution (a_w = 0.22).

The HPLC chromatograms relative to the LPC esterification reactions only showed the presence of *sn*-1,2-PC, however the yield was not total. The products, in particular LPC, could have been lost during the reaction washing with the two different mixtures of $CHCl_3$:MeOH:H_2O or by adsorption and inclusion of reagents/products in the pores of the molecular sieves.

It is important to note that higher yields for the enzymatic acylation of *sn*-2-LPC were obtained when compared to those of analogous reactions reported in the literature (HOSOKAWA *et al.*, 1995).

2b. Chemical acylation of *sn*-2-LPC to synthesize *sn*-1,2-PC

The chemical approach (Section 2b) for the esterification of *sn*-2-LPC to obtain PC showed higher yields in comparison with the enzymatic ones; the reactions were carried out using DCCD as a coupling-condensation agent and DMAP as a catalyst. The chemical method has many advantages, such as simplicity, rapidity and low cost and the absolute yield increased to 65% (relative yield = 95%), at room temperature overnight.

CONCLUSIONS

This work, together with a previous study on GPC enzymatic production from *sn*-1,2-PC (BLASI *et al.*, 2006), reports a method for the enzymatic synthesis of structured *sn*-1,2-PC using natural and inexpensive PL as starting material.

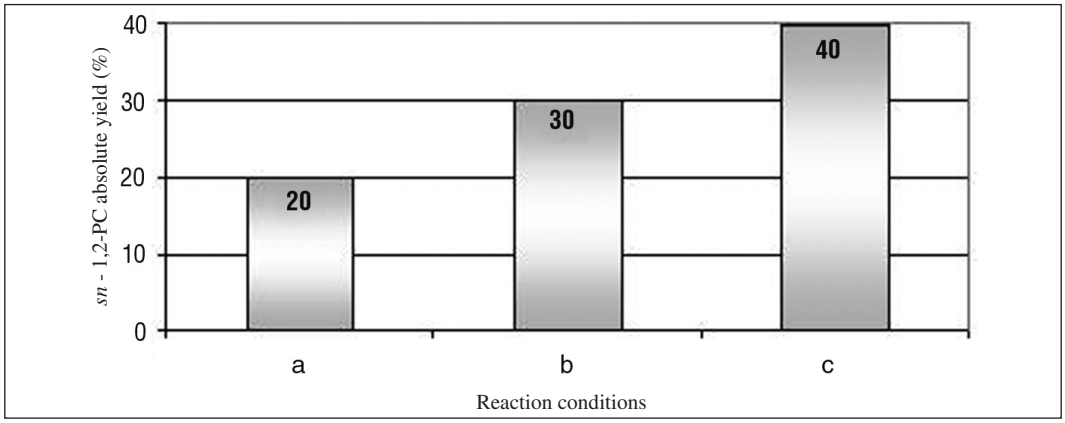


Fig. 3 - Absolute yields (%) of *sn*-1,2-PC, obtained by *sn*-2-LPC esterification: a) without dimethylformamide, b) with dimethylformamide and molecular sieves and c) with dimethylformamide (0.1 mL), under controlled a_w (0.22); the standard deviation values were never higher than 4.6% (n=3).

The acylation of GPC using FA vinyl esters and *C. antarctica* lipase was selective for the formation of *sn*-2-LPC, with a reaction time of 22 h. The yield was improved by using a large excess of "activated" FA and by the addition of *t*-butanol; the yields changed in response to reaction temperature and FA chain length.

The esterification of *sn*-2-LPC in glycerol and in the presence of dimethylformamide gave an absolute yield of about 30%. This value increased to about 40% when the reaction medium was equilibrated overnight with a saturated solution of CH_3COOK (a_w=0.22). Better PC yields were obtained by chemical acylation of *sn*-2-LPC.

The *sn*-1,2-PC produced could be used as starting compounds to obtain structured *sn*-1,2-diacylglycerols and, with a successive step, structured triacylglycerols (TAG), thus reproducing the biosynthesis of natural TAG.

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Revised paper received July 18, 2007 Accepted September 11, 2007

PAPER

CHARACTERIZATION OF TRIACYLGLYCEROLS IN *ARBUTUS UNEDO* L. SEEDS

CARATTERIZZAZIONE DEI TRIACILGLICEROLI IN SEMI DI *ARBUTUS UNEDO* L.

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ABSTRACT

Arbutus unedo L. (fam: Ericaceae), a typical plant of the Mediterranean woods, produces many reddish berries with small seeds. This study was carried out to characterize the triacylglycerol (TAG) fraction of the oil extracted

RIASSUNTO

Arbutus unedo L. (fam: Ericaceae), tipica pianta della macchia mediterranea, produce numerose bacche rossastre con piccoli semi. La presente ricerca è stata condotta al fine di caratterizzare la frazione triacilglicerolica (TAG)

- Key words: *Arbutus unedo*, Ag^+-HPLC, lipids, stereospecific analysis, triacylglycerols -

from the seeds of *A. unedo* berries by stereospecific analysis and by a procedure based on hyphenated techniques (silver ion high-performance liquid chromatography -Ag^+-HPLC- and stereospecific analysis of some sub-fractions). *A. unedo* lipids have a notable amount of essential fatty acids (EFA) and low levels of saturated fatty acids; furthermore there is a marked asymmetry in the FA positional distribution on the TAG glycerol backbone.

dell'olio estratto dai semi delle bacche di *A. unedo* sia mediante analisi stereospecifica sia con hyphenated techniques (tecniche collegate, nel caso specifico costituite da cromatografia di argentazione -Ag^+-HPLC- e analisi stereospecifica chimico-enzimatica di alcune sub-frazioni). I lipidi della matrice considerata presentano un significativo contenuto di acidi grassi essenziali (EFA) e bassi livelli di FA saturi; inoltre, è stata rilevata una notevole asimmetria nella distribuzione degli FA nello scheletro glicerolico.

INTRODUCTION

Strawberry tree (*Arbutus unedo* L., fam: Ericaceae) is a typical plant of the Mediterranean woods. Its name is probably attributable to Virgil; in fact it seems that the poet derived the name *Arbutus* from the latin words "*arbustus*" and *unum edo* from "unum edo". Strawberry tree is an evergreen shrub or small tree of average size, with twisting, reddish branches, alternating lanceolate serrate leaves and flowers clustered in racemes at the end of the branches. It produces many spherical reddish berries, with a yellow or orange sweet delicate pulp that tastes like strawberry; the berry contains many small seeds whose composition deserves study (SCORTICHINI, 1986; 1990).

The *Arbutus* genus is distributed throughout southern Europe, but is also present in some areas of Africa, Asia, Canada and northwestern America. *A. unedo* found in the Mediterranean area is a spontaneous species of great landscaping and ecological value (SEIDEMANN, 1995). Regular consumption of strawberry tree berries is desirable, because the composition of the fruit is quite similar to many of the more widely consumed fruits, particularly when the sugar (14% fresh wt) and vitamin C (150-280 mg/100 g fresh wt) contents are considered (AYAZ *et al.*, 2000; ALARCÃO-E-SILVA *et al.*, 2001). Other studies on strawberry tree have reported on the lipid (MELETIOU-CHRISTOU *et al.*, 1994), essential oil (KIVCAK *et al.*, 2001), volatile compound (YAYLI *et al.*, 2001) and flavonoid (MALES *et al.*, 2006) contents of the fruit.

In this study the triacylglycerol (TAG) fraction of the oil extracted from *A. unedo* seeds was studied. The aim was to characterize the TAG fraction, particularly the iso-unsaturated TAG sub-fractions of the strawberry tree seed by stereospecific analysis of the total fraction, as well as by silver ion high-performance liquid chromatography -Ag^+-HPLC- and stereospecific analysis of some sub-fractions.

MATERIALS AND METHODS

Materials

Sampling

Strawberry tree (*A. unedo* L.) fruits were obtained from young trees in central Italy (Umbria and Lazio) in November 2005. The berries were firm, spherical, bright scarlet-red, with a diameter

of 1-1.5 cm. Five samples of *A. unedo* berries were randomly collected (about 1 kg for each sample). The oil samples were obtained from the milled and homogenized seeds by Soxhlet extraction with *n*-hexane as solvent; the organic extracts were pooled, treated with anhydrous Na_2SO_4 and the organic solvent was evaporated.

Chemicals and reagents

All the solvents and reagents, Analar or HPLC grades, were supplied by Sigma-Aldrich (St. Louis, MO).

Methods

Fatty acid (FA) composition (%) of the TAG fraction by high resolution gas chromatography

The TAG fraction was separated from *A. unedo* seed samples by thin layer chromatography TLC as previously described (NERI *et al.*, 1998), using silica gel plates, 0.25 mm, 20 cm x 20 cm (Macherey-Nagel, Düren, Germany) and a mixture of petroleum ether 40-60°C/diethyl ether/formic acid (70/30/1, v/v/v) as eluent.

The methyl ester derivatives of the constituent fatty acids (FAME) were prepared by sodium methoxide-catalyzed transesterification, in the presence of an internal standard, methyl nonadecanoate, to quantify the sub-fractions, previously separated by Ag^+-HPLC (SANTINELLI *et al.*, 1992). A Chrompack 9001 gas chromatograph (Chrompack International B.V., Middelburg, The Netherlands), equipped with a split/splitless injection system and a flame ionization detector was used for FAME analysis; the separation was obtained using a Supelcowax 10 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Supelco, Bellefonte, PA). The oven temperature was maintained at 165°C for 3 min, then raised 3°C/min to 240°C and held for 10 min. Helium was the carrier gas at a flow rate of 2 mL/min. A MOSAIC integration software (Chrompack International B.V.) was used for data acquisition and processing.

Ag^+-HPLC analysis

The HPLC analyses were carried out using a solvent delivery system Model LC-10AD VP (Shimadzu, Kyto, Japan) equipped with a light-scattering detector (Model DDL21, Cunow SA, Cergy St. Cristophe, France). The chromatograms were acquired and the data were analyzed using the Shimadzu Class-VP software.

The chromatographic separation of TAG was achieved with a Ag^+ Chrom-Spher 5 Lipids (5 μm; 250 mm × 4.6 mm) column (Chrompack International B.V.) operating at ambient temperature.

An adjustable stream-splitter was installed between the column and the detector.

The analyses were carried out using a ternary solvent gradient system: (A) 1,2-dichloroethane/dichloromethane (1:1, v/v); (B) acetone; (C) acetone/acetonitrile (9:1, v/v). The analysis conditions were: from 100% A to 60% A - 40% B for 18 min and then to 90% B - 10% C for 25 min; the flow rate was 1.0 mL/min (SANTINELLI *et al.*, 1992).

Stereospecific analysis of TAG

Pancreatic lipase hydrolysis was carried out on TAG to obtain *sn*-2-monoacylglycerols (*sn*-2-MAG), according to the NGD method (1989); the HRGC analysis of FAME of *sn*-2-MAG gave the FA % composition at the *sn*-2- position (A_2). The preparation of *sn*-1,2-phosphatidic acids (*sn*-1,2-PA) was carried out by *sn*-1,2-diacylglycerol kinase procedure (DAMIANI *et al.*, 1994a,b); the HRGC analysis of FAME of *sn*-1,2-PA gave the FA % composition at the *sn*-1,2- positions ($A_{1,2}$).

The FA composition at the *sn*-1- and *sn*-3- positions was obtained using the

% FA compositions of *sn*-1,2-PA, *sn*-2-MAG and total TAG (At), applying the following formulae:

$$A_1 = 2 \cdot A_{1,2} - A_2$$
$$A_3 = 3 \cdot At - A_2 - A_1$$

Statistical analysis

Five replications of each analytical determination were performed. The values are reported as the mean value ± standard deviation.

RESULTS AND DISCUSSION

The TAG fraction, purified from *A. unedo* lipids (11.1% of seeds), was analyzed for its FA composition and the stereospecific distribution. Subsequently, Ag^+-HPLC analysis was used to separate the TAG fraction according to the degree of unsaturation (CHRISTIE, 1982), and the major iso-unsaturated sub-fractions (> 3%) were further characterized.

Table 1 shows the FA composition of the total lipids and the TAG fraction from the five *A. unedo* seed samples. The results show that the total lipids and TAG fraction were made up of low levels of saturated FA (SFA, about 14.0%) and high levels of monounsaturated (MUFA, about 41.1%) and polyunsaturated ones (PUFA, about 45.0%). Strawberry tree seed oil is of particular interest because of its high essential FA (EFA) content, with 18.7% linoleic (L, C18:2n-6) and 26.3% α-linolenic (Ln, C18:2n-3) acids.

Table 1 - Fatty acid composition of total lipids and TAG fraction of *A. unedo* seed oil.

	A. unedo seed oil	
	Total lipids	TAG fraction
C 14:0	0.3±0.07	0.3±0.06
C 16:0	9.7±0.32	9.6±0.15
C 16:1n-7	0.6±0.14	0.7±0.09
C 18:0	3.6±0.19	3.5±0.08
C 18:1n-9	40.1±1.08	40.1±0.12
C 18:2n-6	18.7±0.22	18.6±0.28
C 18:3n-3	26.3±0.29	26.5±0.11
C 20:0	0.4±0.09	0.3±0.04
C 20:1n-9	0.3±0.12	0.3±0.06
SFA	14.0	13.7
MUFA	41.0	41.1
PUFA	45.0	45.1

mean values, mol % ± standard deviations (n=5).

Table 2 - Positional fatty acid composition of TAG fraction from *A. unedo* seed oil.

	sn-1-	*sn*-2-	*sn*-3-
C 14:0	0.4±0.04	0.1±0.00	0.3±0.15
C 16:0	19.3±0.19	0.5±0.11	8.9±0.56
C 16:1n-7	1.1±0.07	0.3±0.13	0.7±0.27
C 18:0	6.8±0.35	0.2±0.05	3.6±0.45
C 18:1n-9	38.4±0.27	49.2±0.15	32.7±0.52
C 18:2n-6	15.2±0.26	22.6±0.07	18.1±0.94
C 18:3n-3	17.7±0.16	26.8±0.13	35.0±0.35
C 20:0	0.3±0.09	0.2±0.04	0.4±0.14
C 20:1n-9	0.7±0.10	0.1±0.02	0.1±0.03
SFA	26.8	1.0	13.2
MUFA	40.2	49.6	33.5
PUFA	32.9	49.4	53.1

mean values, mol % ± standard deviations (n=5).

The FA positional composition of the TAG fraction, obtained from stereospecific analysis, is reported in Table 2. The *sn*-2- position was almost entirely (99.0%) esterified with unsaturated FA, 49.6% MUFA and 49.4% PUFA. Only 1.0% SFA were found at this position, as generally observed in vegetable oils (CHRISTIE, 1987). Oleic acid (O; C18:1n-9) was the most abundant FA (49.2%) in the *sn*-2- position, followed by EFA (L 22.6%; Ln 26.8%).

The results of stereospecific analysis showed an asymmetry in the distribution of the FA between the *sn*-1- and *sn*-3- positions of the TAG fraction. For example, palmitic (P, C16:0) and stearic (S, C18:0) acids had similar

Table 3 - Major iso-unsaturated TAG sub-fractions of *A. unedo* seed oil (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

TAG sub-fraction	mol (%)
MMT	13.5
MDT	12.1
SMT	9.8
MMD	9.2
MTT	8.4
SMM	7.2
SMD	6.7
SDT	4.6
MDD	4.2
DTT	3.7
STT	3.2

mean values, mol %; n=5.

trends, showing a marked preference for the *sn*-1- position. There was a particular positional composition for PUFA; linoleic acid was preferably located in the *sn*-2- position (22.6%) and less in the *sn*-3- and *sn*-1- positions (18.1% and 15.2%, respectively). Linolenic acid was preferably esterified in the *sn*-3- position (35.0%), with 26.8 and 17.7% in the *sn*-2- and *sn*-1- positions, respectively.

Table 3 shows the proportions of the major (> 3%) TAG sub-fractions separated by Ag^+-HPLC analysis. The TAG sub-fractions were also quantitatively evaluated by using a home-made software that processes the results of positional composition of FA to provide information about the contents of all the TAG molecular species.

The stereospecific analysis was carried out on the most representative TAG sub-fractions, MMT, MDT, SMT, MMD, MTT and SMM (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA); their positional compositions are reported in Table 4. A marked asymmetry of the FA distribution in the

Table 4 - Positional fatty acid composition of the iso-unsaturated sub-fractions: MMT, MDT, SMT, MMD, MTT and SMM (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

	MMT			MDT			SMT		
	sn-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C14:0							1.1	0.2	0.5
C16:0							51.7	1.0	17.7
C16:1n-7	2.1	0.4	0.9	1.2	0.2	0.4	0.4	0.3	0.5
C18:0							18.2	0.3	7.1
C18:1n-9	74.9	72.7	47.3	40.7	34.7	21.9	15.1	58.6	24.5
C18:2n-6				33.2	37.5	29.4			
C18:3n-3	21.7	26.7	51.6	24.2	27.5	48.2	12.3	39.0	48.7
C20:0							0.9	0.5	0.8
C20:1n-9	1.3	0.1	0.2	0.7		0.1	0.3	0.1	0.1
	MMD			MTT			SMM		
	sn-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C14:0							1.0	0.2	0.7
C16:0							44.5	0.9	24.6
C16:1n-7	2.0	0.4	1.2	1.2	0.2	0.4	1.0	0.5	1.2
C18:0							15.7	0.3	9.9
C18:1n-9	79.4	66.3	59.2	42.8	36.1	18.4	36.5	97.5	62.2
C18:2n-6	27.5	33.2	39.4						
C18:3n-3				55.2	63.6	81.2			
C20:0							0.8	0.4	1.1
C20:1n-9	1.2	0.1	0.2	0.7		0.1	0.6	0.2	0.2

three *sn*- positions on the glycerol backbone was observed for all the major sub-fractions.

The results showed that in the MMT, MDT, MMD and MTT fractions all the MUFA were preferably located at the *sn*-1- position. Oleic acid, the most abundant FA, showed the same trend in all these fractions and was preferentially esterified in the *sn*-1- position, followed by the *sn*-2- and *sn*-3- positions. Oleic acid preferred the *sn*-2- position in the SMT and SMM fractions (58.6% and 97.5%, respectively). PUFA showed a particular distribution in all the cited fractions except for SMM. Linoleic acid was preferably located at the *sn*-2- position (37.5%) in the MDT fraction but it was located at the *sn*-3- (39.4%) in the MMD fraction. Linolenic acid was the most abundant acid in the *sn*-3- position of the MMT, MDT, SMT and MTT sub-fractions. The SFA in the SMM fraction were located mainly at the *sn*-1- position, followed by the *sn*-3- position.

Some of the molecular isomeric TAG species of the major sub-fractions are shown in Fig. 1. The results showed the presence of different levels of isomeric and enantiomeric TAG species. For example, the ratio of enantiomeric species *sn*-OOLn (6.6%) and *sn*-LnOO (2.8%) was about 2.3 in the MMT sub-fraction. This ratio, together with others of different TAG sub-fractions, could serve as markers to characterize *A. unedo* seed. Furthermore, the TAG species with EFA in the *sn*-2- position could also be of interest from a nutritional point of view. For example, the *sn*-OLnO made up 3.4% of the total TAG in the MMT sub-fraction. In the MDT sub-fraction, about 4.3% of the TAG species (*sn*-OLLn and *sn*-LnLO) had linoleic acid at the *sn*-2- position and about 3.2% of the TAG species (*sn*-OLnL and *sn*-LLnO) had linolenic acid at this position. In contrast in the SMT sub-fraction, about 2.5% of the TAG species (*sn*-PLnO and *sn*-OLnP) had linolenic acid at the *sn*-2-position.

These results support the hypothesis that every natural oil has its own typical FA positional distribution and could be considered as the "fingerprint" of the TAG fraction of the oil. Since dietary lipids are absorbed as *sn*-2-MAG and as free FA, produced by lipase hydrolysis (BRINDLEY, 1985), the positional distribution of FA on the glycerol backbone has an important nutritional impact. Consequently, the stereospecific analysis is also an important tool for determining the nutritional quality of TAG. Furthermore, the enantiomeric ratios between the TAG molecular species could be of considerable importance as markers to distinguish the lipids derived from different species and varieties.

When the composition of strawberry tree seed oil was compared with that of blueberry (*Vaccinium corymbosum*, fam: Ericaceae) (PARRY *et al.*, 2005), *A. unedo* seed oil had a higher oleic acid content, lower linoleic acid content and a similar linolenic acid level, with a n-6/n-3 PUFA ratio of about 0.7. This ratio is the lowest one, if compared with those of other berries. Since the recommended n-6/n-3 FA ratio is estimated to be 4/1, while the current dietary n-6/n-3 FA ratio is about 10/1 (KRIS-ETHERTON *et al.*, 2000), new dietary sources with low n-6/n-3 PUFA ratios are in high demand for improving human nutrition.

CONCLUSIONS

The present study shows that strawberry tree seed oil is characterized by a low SFA content, a high oleic acid content, a significant presence of n-6 and n-3 EFA and a low n-6/n-3 FA ratio. The results of stereospecific analysis of the TAG fraction show marked asymmetry in the FA distribution among the three *sn*-positions. The high incorporation of EFA in the *sn*-2- position is very important from a nutritional point of view. The Ag^{+}-HPLC analy-

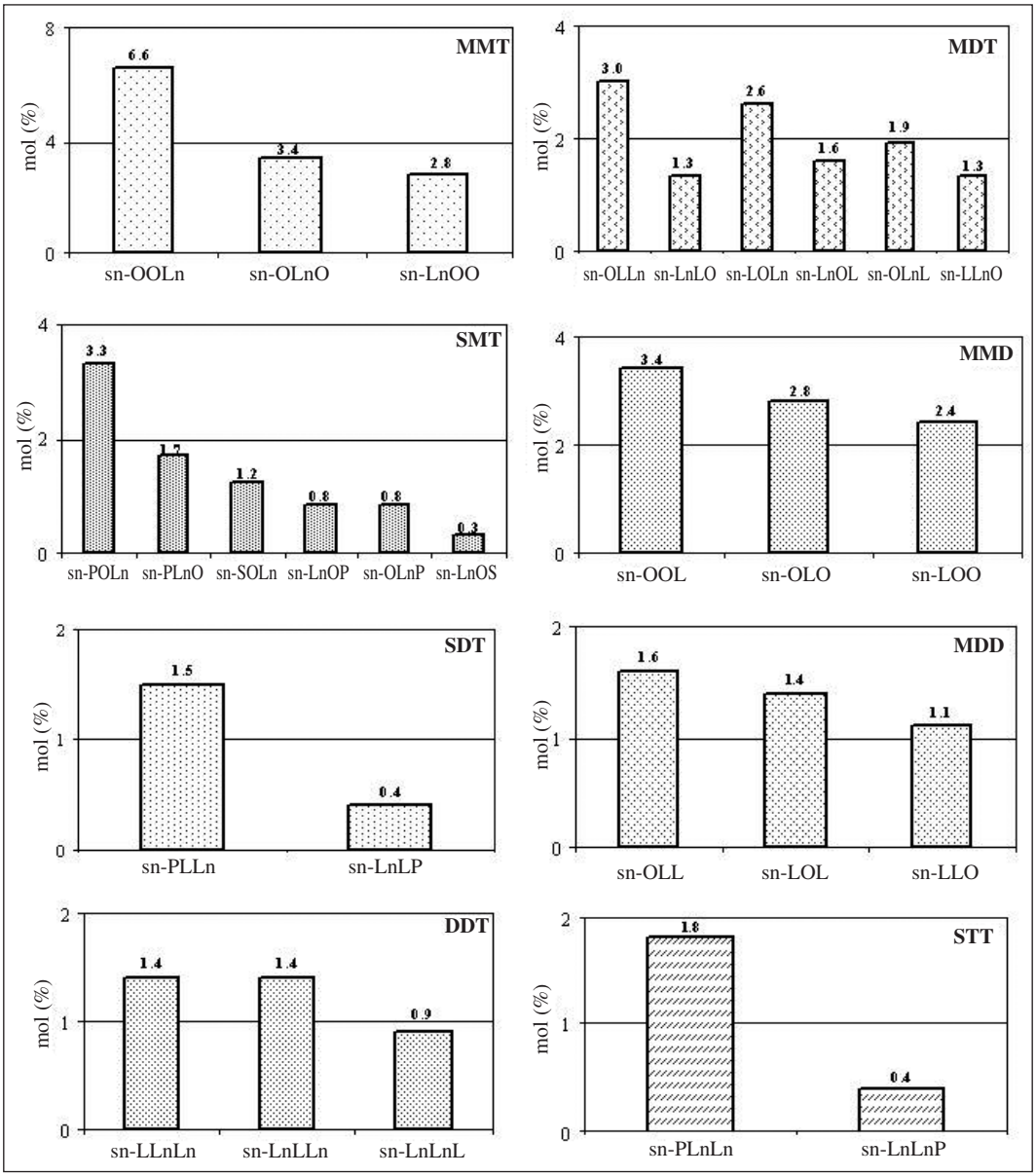


Fig. 1 - Major TAG molecular species of iso-unsaturated TAG sub-fractions of *A. unedo* seed oil: MMT, SMT, SDT, DTT, MDT, MMD, MDD and STT (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

sis of TAG, coupled with stereospecific analysis give other structural information about the lipid fraction. These data could be used to select specific iso-unsaturated sub-fractions, important for their nutritional, physiological and analytical aspects and for industrial applications.

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Revised paper received April 26, 2007 Accepted May 18, 2007

PAPER

STUDY OF PROTEOLYSIS IN MINIATURE TOMA PIEMONTESE CHEESE MADE USING WILD BACTERIA

STUDIO DELLA PROTEOLISI IN FORMAGGI MINIATURIZZATI DI TOMA PIEMONTESE PRODOTTI USANDO BATTERI AUTOCTONI

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ABSTRACT

The effects of 35 strains of indigenous bacteria on proteolysis in model Toma Piemontese cheese were studied at 30 and 60 days of ripening. Proteolysis was assessed by urea-polyacrylamide gel electrophoresis (PAGE) of the pH 4.6-insoluble fractions of cheese, by reversed-phase high performance liquid chromatography (RP-HPLC) of the pH 4.6-soluble fractions at 30 and 60 days of ripening and by free amino acid (FAA) analysis at 60 days of ripening. Urea-PAGE showed that α_{s1}-casein was hydrolysed more than β-casein. The use of

RIASSUNTO

In questa ricerca è stato studiato l'effetto prodotto dall'utilizzo di 35 ceppi di batteri lattici autoctoni sulla proteolisi in formaggi modello di Toma Piemontese. La proteolisi è stata valutata a 30 e a 60 giorni attraverso l'elettroforesi su gel di urea-poliacrilamide (Urea-PAGE) della frazione insolubile a pH 4,6 e la cromatografia liquida ad alte prestazioni in fase inversa (RP-HPLC) della frazione solubile a pH 4,6 nonché attraverso l'analisi degli amino acidi liberi (FAA) sui formaggi a 60 giorni di stagionatura. L'urea-PAGE ha mostrato

- Key words: indigenous strains, proteolysis, Toma Piemontese cheese -

single strains markedly influenced the peptide profiles obtained by RP-HPLC of the cheeses. Significant differences in the levels of FAAs were also observed among the cheeses. The results of this study highlighted marked differences in the acidification and proteolytic activities of these bacterial strains.

come l'α_{s1}-caseina sia stata idrolizzata di più rispetto alla β-caseina, mentre la RP-HPLC ha evidenziato l'effetto dei diversi ceppi batterici utilizzati sui profili peptidici. Significative differenze sono state rilevate inoltre nel livello di FAA e ciò conferma la differente capacità proteolitica dei ceppi saggiati.

INTRODUCTION

In ripening, cheese is subjected to three major categories of biochemical events: glycolysis, lipolysis and proteolysis (FOX, 1989). However, proteolysis is the most important process that occurs during ripening because it contributes to texture through the hydrolysis of the protein matrix and to flavour and off-flavour formation as a direct consequence of the production of peptides and free amino acids and indirectly by the liberation of amino acids that can be subjected to other changes including decarboxylation, deamination or desulphuration to produce other flavour compounds (SOUSA *et al.*, 2001).

Proteolysis in cheese is catalysed by proteinases and peptidases derivated from six sources: coagulant (chymosin, pepsin, fungal acid proteinase or plant acid proteinases); milk (plasmin, cathepsin D and other somatic cell proteinases); starter lactic acid bacteria; adventitious non-starter lactic acid bacteria; addition of secondary starters in some cheese varieties (*Penicillium roqueforti*, *Penicillium camemberti*, *Geotrichum candidum*) and exogenous proteinases and/or peptidases which may be added to accelerate ripening or to accentuate flavour of the cheese (FOX and McSWEENEY, 1996; UPADHYAY *et al.*, 2004). Proteolysis in cheese can be divided into two phases: primary proteolysis which is the initial hydrolysis of caseins by the coagulant and the milk proteinases to produce the large and intermediate-sized peptides that are subsequently degraded during secondary proteolysis. During primary proteolysis, chymosin, the principal enzyme in rennets used in cheesemaking, acts on α_{s1}-casein at Phe_{23}-Phe_{24}. Plasmin, the indigenous alkaline milk proteinase, cleaves the β-casein to give γ-caseins (β-CN f29-209, β-CN f106-209 and β-CN f108-209). The products of the hydrolysis of α_{s1}- and β-caseins can be detected by polyacrylamide gel electrophoresis (urea-PAGE) (GRAPPIN *et al.*, 1985). During secondary proteolysis enzymes derived from the starter and non-starter flora of cheese act on the products of primary proteolysis and produce small peptides and free amino acids. These products can be extracted as the water-soluble fractions and analysed by reversed-phase high performance liquid chromatography (RP-HPLC) (RANK *et al.*, 1985; ARDÖ and GRIPON, 1991; McSWEENEY and FOX, 1997).

Lactic acid bacteria are very important for dairy production; they have a very complex proteolytic system composed of a proteinase and a wide range of peptidases that are responsible for the formation of small peptides and amino acids in cheese. The proteolytic systems of *Lactococcus* spp. and of *Lactobacillus* spp. have been studied in great detail because of their economic value as starter cultures in dairy fermentations (NORAINI and ELMER, 1990; BOCKELMANN,1995).

Bacteria which take part in the differ-

ent biochemical processes that occur in cheese during ripening come from starters added during cheesemaking or from the environment but the former are the most important as they are deliberately added and increase to high numbers.

In many European countries, cheeses (e.g. Beaufort, Comté, Grana Padano and Parmigiano Reggiano) are traditionally made using natural cultures obtained by incubating milk or whey from the previous day's production under defined conditions. The inoculum is characterised by a well-balanced flora of mesophilic and thermophilic lactic acid bacteria. Nevertheless, natural cultures are characterised by a high variability in strain composition over time and require a long time to be prepared. It is now normal practice to make cheese using commercial starter cultures that are easy to use; the cheeses that are produced are standardised but with some organoleptic impoverishment (GRAPPIN and BEUVIER, 1997; MENÉNDEZ *et al.*, 2004). As a consequence, interest has been shown in the genotypic and technological characterisation of wild isolates from artisanal cheeses because new strains could be selected and used as defined-strain cultures to give cheeses with technological and organoleptic characteristics that are more similar to traditional cheese (COGAN *et al.*, 1997; SHAKEEL-UR-REHMAN *et al.*,1999; BERESFORD *et al.*, 2001; GONZALES *et al.*, 2003; POVEDA *et al.*, 2003; HAYALOGLU *et al.*, 2004; DI CAGNO *et al.*, 2006).

In Italy, these autochthonous starter cultures are currently being used to produce many Protected Denomination of Origin (PDO) cheeses such as Asiago, Bitto, Fontina and Pecorino Toscano but they have not been used for Toma Piemontese which is the most important cheese from the northwestern Alps.

Toma Piemontese PDO cheese was traditionally made in mountain farms using raw milk. However, over the last decade, it has been manufactured on a large scale in small and large dairy plants using pasteurized milk with the addition of industrial freeze-dried starters that generally contain mesophilic lactic acid bacteria. Toma Piemontese is a cylindrical, semi-hard, semi-cooked cheese. It is produced in two sizes, large (6-8 kg, 24-34 cm in diameter) and small (1.8-5.9 kg, 15-25 cm in diameter). The milk is cooled to 37°C and the rennet is added at a concentration of 0.15-0.20 mL × L^{-1}. The coagulation time is established visually by the producer. The curd is then cut into 5-10 mm pieces, placed in a cloth, pressed, drained for 24 h and then salted. Cheese is ripened in a cold room or caverns at 6-10°C and 85% humidity for 30-60 days.

To date the proteolysis in Toma Piemontese cheese has not been examined nor has the use of single artisanal strains. The objective of the present study was to evaluate the effect of thirty-five strains of LAB isolated from artisanal Toma Piemontese cheeses on proteolysis in model cheeses after 30 or 60 days of ripening.

MATERIALS AND METHODS

Cultures

Lactococcus lactis subsp. *lactis* (A 1.1, A 1.4, A 1.6, A 1.13, A 1.18, A 1.20, A 2.1, A 2.5, TA 1.2, TA 2.1, TA 2.4, TA 2.5, TB 2.1, TB 2.2, TB 2.4, TB 2.6, TB 2.7, TB 2.8, TC 1, TC 6 and TC 8), *Lactococcus lactis* subsp. *cremoris* (A 1.8 and A 2.4), *Lactobacillus paracasei* (TO 1.1 and TA 1.4), *Streptococcus thermophilus* (B 1.18, A 2.3 and TA 1.1), *Streptococcus macedonicus* (TA 3.1, TA 3.4, TA 3.5, TB 1.4 and TB 1.9), *Lactobacillus fermentum* (TA 2.2) and *Lactobacillus casei* subsp. *rhamnosus* (TA 3.3) were used separately as starter cultures. These organisms were isolated from artisanal Toma Piemontese cheeses and characterised as reported by FORTINA *et al.* (2003) and were obtained

from the culture collection of the Department of Exploitation and Protection of the Agricultural and Forestry Resources, Agricultural Microbiology and Food Technologies Section, Agricultural Faculty, University of Turin. Before use, each strain was cultivated in M17 broth (Merck, Darmstadt, Germany) for two transfers at 37°C for 24 h (1% inoculum). For starter propagation, the broth cultures were grown in reconstituted skim milk (10 g × 100 mL^{-1} autoclaved for 10 min at 115°C) with an inoculum of 1%. Before their use as inoculum for cheesemaking, a microscopic count was done on the cultures of each strain in order to standardise the concentration of inoculum added to the milk at 10^7 cfu mL^{-1}.

Cheese manufacture

Model cheeses were made using the method described by SHAKEEL-UR-REHMAN *et al.* (1998) but modified to produce cheeses that were more similar to Toma Piemontese cheese.

No control cheese was included in this study because the only possible control cheeses could have been cheese made from raw milk, a starter-free cheese or the use of a commercial starter but none of these types of cheeses would have been a good control. In the first case raw milk would have had a very heterogeneous microflora which would not have been possible to replicate day by day. Many studies have been conducted on starter-free cheese, and the results have shown that the differences between starter-free and starter cheese is much greater than any possible strain-to-strain differences. The use of a commercial starter as a control would have simply been another strain to be tested. Moreover all the other parameters in cheesemaking were the same for each strain so that the differences in the final cheeses were determined by the different strains used.

For each strain, six model cheeses were made. Raw milk was pasteurised in beakers at 63°C for 30 min. Pasteurised milk was put into plastic centrifuge bottles (200 mL) cooled to 37°C and maintained at this temperature in a water bath. A starter inoculum of 2% was added to milk (corresponding to 10^7 cfu mL^{-1}) which was ripened for 15 min. $CaCl_2$ (132 µL of a 1 mol × L^{-1} solution/200mL milk) was then added and held for 15 min. Rennet (Maxiren-180, DSM, Seclin Cedex, France) was added (43.5 µL/200 mL) and the milk was held for 45 min to obtain a firm coagulum. After the coagulum was cut, it was held for 2 min and then stirred slowly for 10 min using a glass rod. The temperature was then increased to 41°C over 30 min. Curd and whey were then centrifuged at room temperature for 60 min at 1700 *g* in an MSE 'MAJOR' centrifuge (MSE Scientific Instruments, Crawley, Sussex, England). The whey was drained and the curds were covered with 3 mL of sterile water and then kept in the centrifuge bottles at 30°C for 18 hours. The water was drained and the cheeses were inverted in the bottles and then recentrifuged at 1700 *g* for 20 min. The cheeses were then brine salted (20% NaCl, 0.05% $CaCl_2 \times 2H_2O$) for 30 min at room temperature. After salting, cheeses were removed from the bottles wiped with tissue paper and vacuum packed. They were ripened at 8°C for 30 and 60 days. At the end of the ripening period the weight of the miniature cheeses ranged from 20 to 22 g. Cheeses were sampled for compositional analysis on day 30 and for proteolysis on days 30 and 60.

Composition analysis

Cheeses were analysed at 30 days for moisture by the oven drying method at 102°C (IDF, 1982) and for salt by titration with $AgNO_3$ according to the method of BRADLEY *et al.* (1993). The pH was determined at 30 and 60 days by placing a penetration electrode in contact with the cheese mass.

Assessment of proteolysis

After ripening for 30 and 60 days, pH 4.6-insoluble and –soluble fractions were prepared by a slight modification of the method of KUCHROO and FOX (1982) as described by HAYALOGLU *et al.* (2004). Instead of using 20 g of cheese and 40 mL of water for the extraction, 15 g of cheese and 30 mL of water were used.

Urea-polyacrylamide gel electrophoresis (Urea-PAGE) [12% T (total monomer), 4% C (crossing-linking agent as percentage of total monomer), pH 8.9] of the pH 4.6-insoluble fractions of the cheeses was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of ANDREWS (1983) with the modifications of SHALABI and FOX (1987). The gels were stained directly with Coomassie Brilliant Blue G-250 by the method of BLAKESLEY and BOEZI (1977) and destained using distilled water. After destaining, gel slabs were digitised with a scanner (Scanjet 6300C, Hewlett Packard, Houston, USA). Scans of the electrophoretograms were used to quantify bands using densitometric software (Image Master TotalLab 1D Gel analysis v 1.11 software, Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK). The caseins and peptides were quantitatively determined by integration of peak volumes using a densitometer.

Peptides of the pH 4.6-soluble fraction of the cheese were determined by RP-HPLC using the method described by HAYALOGLU *et al.* (2004).

For the free amino acid (FAA) analysis, the pH 4.6-soluble fractions of the cheeses were deproteinised by mixing them with an equal volumes of 24% (w/v) trichloroacetic acid (TCA). The solutions were allowed to stand for 10 min before centrifugation at 14400 *g* for 10 min (MSE, Microcentaur, DJB Labcare Ltd, Newpost Pagnell Buckinghamshire, UK). The supernatant was removed and diluted with 0.2 M sodium citrate buffer (pH 2.2) to give approximately 250 nmol of each amino acid residue. The samples were then diluted 1:2 with the internal standard norleucine to give an approximate final concentration of 125 nm of each amino acid residue in 1 mL of solution. Samples were then analysed using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation-exchange column. The individual free amino acids were separated by ion-exchange chromatography with post-column ninhydrin derivatization and visible colorimetric detection at 570 nm. Results were obtained using a Aminotaq data handling system (J & C Joel Ldt, Halifax, UK).

Statistical analysis

Two cheeses for each strain were used for each analysis. The data used for the statistical analysis were the means of these two replicates.

Data from urea-PAGE gels of the pH 4.6-insoluble fractions were analysed using multivariate statistical analysis. Similar bands in the urea-PAGE gels were recognised visually as described by McSWEENEY *et al.* (1994) and the peak volume of corresponding bands were used as data for cluster analysis (CA). Data from RP-HPLC of the pH 4.6-soluble fractions were analysed using CA to evaluate the effect of the single-strain starters. Data for the statistical analysis of the RP-HPLC chromatograms were obtained by visually recognising similar peaks in the chromatograms and using peak heights as variables as described in PRIPP *et al.* (1999). The peak heights were obtained by converting the corresponding chromatogram to an ASCII file. CA was performed by using Euclidean distance without standardising the variables. Statistical analyses were performed using Statistica for Windows (StatSoft for Windows Ver. 7, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Compositional analysis

After 30 days of ripening, the range of compositional parameter values of all the cheeses analysed were: 56-57% moisture, 1.5-1.6% salt and pH 5.11. These values are close to those of Toma Piemontese cheese (moisture 46-54%, salt 0.8-3.5 and pH 5.00-5.52) (AMBROSOLI *et al.*, 1998).

Urea-PAGE

Urea-PAGE electrophoretograms of the pH 4.6-insoluble fractions of cheese samples after 30 days of ripening are shown in Fig. 1. The peptide profiles obtained by the urea-PAGE were the result of the direct effects of the rennet and indigenous milk enzymes and the effects of pH on the activities caused by some indirect effects of the strains used as starter. The lactic acid bacteria used as starters had different acidification kinetics that resulted in different final pH values in the cheeses in which they were used as starters (Table 1). These differences in pH affected the breakdown of the caseins. The patterns of proteolysis were almost identical in all the cheeses. It can be seen that neither β-casein nor α_{s1}-casein were degraded very extensively. The cheese made with the *Lactobacillus paracasei* TA1.4 and *Streptococcus macedonicus* TA 3.4 strains showed the highest degradation of β-casein followed by those made with *Lactococcus lactis* subsp. *lactis* TA 2.5 and *Streptococcus macedonicus* TA 3.5. These cheeses had a pH of 5.30, 5.29, 5.25 and 5.24, respectively, which would have favoured plasmin action. With respect to γ-caseins, the polypeptides produced by the action of plasmin on β-casein, γ_3-casein

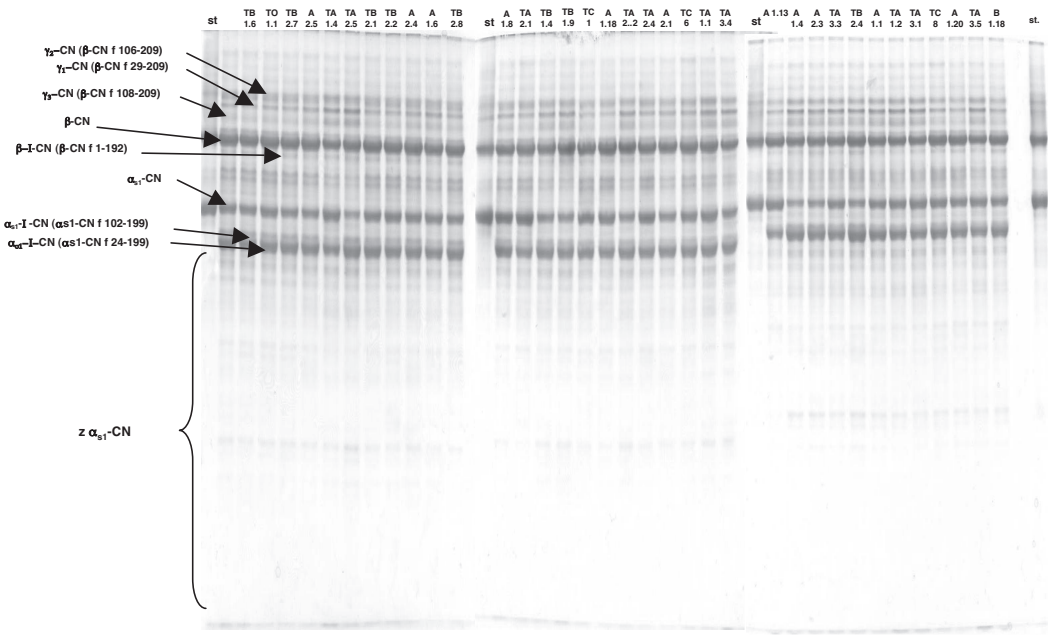


Fig. 1 - Urea-polyacrylamide gel electrophoretograms of miniature Toma Piemontese cheeses made by using different single strains of indigenous bacteria after 30 days of ripening. st = standard Na-caseinate.

Table 1 - pH value of the cheeses made by using single starter strains of lactic acid bacteria after 30 and 60 days of ripening.

Strains	A 1.13	A 1.18	A 1.20	B 1.18	A 1.8	TO 1.1	TA 2.5	TB 2.4	A 2.1	TA 1.2	TB 1.4	TB 1.9
30 days ripening	5.16	5.11	5.24	5.02	5.05	4.90	5.25	5.19	5.12	5.14	5.09	5.09
60 days ripening	5.08	5.04	5.08	4.91	5.04	4.90	5.04	5.06	5.11	5.03	5.08	4.86
Strains	TC 6	TA 3.4	TA 2.2	TA 3.3	A 1.6	A 1.1	A 1.4	A 2.5	TA 2.1	TA 2.4	TB 2.1	TB 2.2
30 days ripening	5.13	5.29	5.11	5.19	5.04	5.20	5.16	5.01	5.06	5.11	5.02	5.02
60 days ripening	5.06	5.10	5.10	5.25	5.03	5.15	5.16	5.00	5.04	5.09	5.01	5.00
Strains	TB 2.6	TB 2.7	TB 2.8	TC 1	TC8	A 2.3	A 2.4	TA 1.1	TA 3.1	TA 3.5	TA 1.4	
30 days ripening	4.86	4.93	5.04	5.09	5.23	5.16	5.03	5.14	5.25	5.24	5.30	
60 days ripening	4.86	4.90	5.03	5.09	5.10	5.14	5.01	5.12	5.20	5.22	5.17	

was present at low concentrations in all the cheeses except for the one made using the *Lactococcus lactis* subsp. *lactis* TA 1.2 strain. γ_2-Casein was present in the highest concentrations in 26 cheeses of the 35 cheeses analysed, most of which were made using *Lactococcus lactis* subsp. *lactis*. γ_1-Casein was present in the highest concentrations in 8 cheeses which were produced by using the *Lactococcus lactis* subsp. *lactis* A 1.4, A 2.3, TB 2.4, A 1.1, *Streptococcus macedonicus* TA 3.1, TA 3.5, B 1.18 and *Lactobacillus casei* subsp. *rhamnosus* TA 3.3 strains. A faint band that corresponded to β-I-casein (β-CN f 1-192), derived from the action of chymosin on β-casein, was present in the electrophoretograms of all the cheeses.

The cheeses made using *Lactococcus lactis* subsp. *lactis* TA 2.5, A 1.4, TB 2.4 and *Streptococcus thermophilus* A 2.3 strains were characterised by the highest degradation of α_{s1}-casein. In all the electrophoretograms, the band corresponding to the α_{s1}-I-casein (α_{s1}-CN f 24-199), which is the product of rennet action on α_{s1}-casein, was present. The highest concentrations of this peptide were obtained with *Lactococcus lactis* subsp. *lactis* TA 2.5, TB 2.4, *Lactococcus lactis* subsp. *cremoris* A 1.8 and *Streptococcus macedonicus* TB 1.4 strains. In all the cheeses, other peptides (marked as z α_{s1}-CN on the electrophoretograms shown in Fig. 1) were present with electrophoretic mobilities that were faster than α_{s1}-I-casein; they may have been derived through the action of rennet and indigenous milk proteinases.

Urea-PAGE electrophoretograms of the pH 4.6-insoluble factions of cheese samples after 60 days of ripening are shown in Fig. 2. After 2 months of ripening, the β-casein was less hydrolysed than α_{s1}-casein. Many authors have discussed the fact that β-casein is more resistant to hydrolysis than α_{s1}-casein during ripening (FOX *et al.*, 1993; SARANTINOPOULOS *et al.*, 2002; HAYALOGLU *et al.*, 2004; FALLICO *et al.*, 2006). γ_1-Casein was present in the highest concentration among the γ-caseins in all cheeses except those that were made using *Lactococcus lactis* subsp. *lactis* TB 2.8 and TC 1 in which γ_3-casein and γ_2-casein were present in the highest concentrations, respectively. Moreover, all the cheeses were characterised by a faint band corresponding to β-I-casein (β-CN f 1-192). With respect to α_{s1}-casein, all the 60-day-old cheeses were characterised by a greater amount of hydrolysis than the 30-day-old cheeses. The strains that degraded this casein the most were *Lactococcus lactis* subsp. *lactis* A 2.5, TA 2.5, TB 2.1, TB 2.4 and *Streptococcus thermophilus* A 2.3. The cheeses that contained the highest concentrations of α_{s1}-I-casein (α_{s1}-CN f 24-199) were those

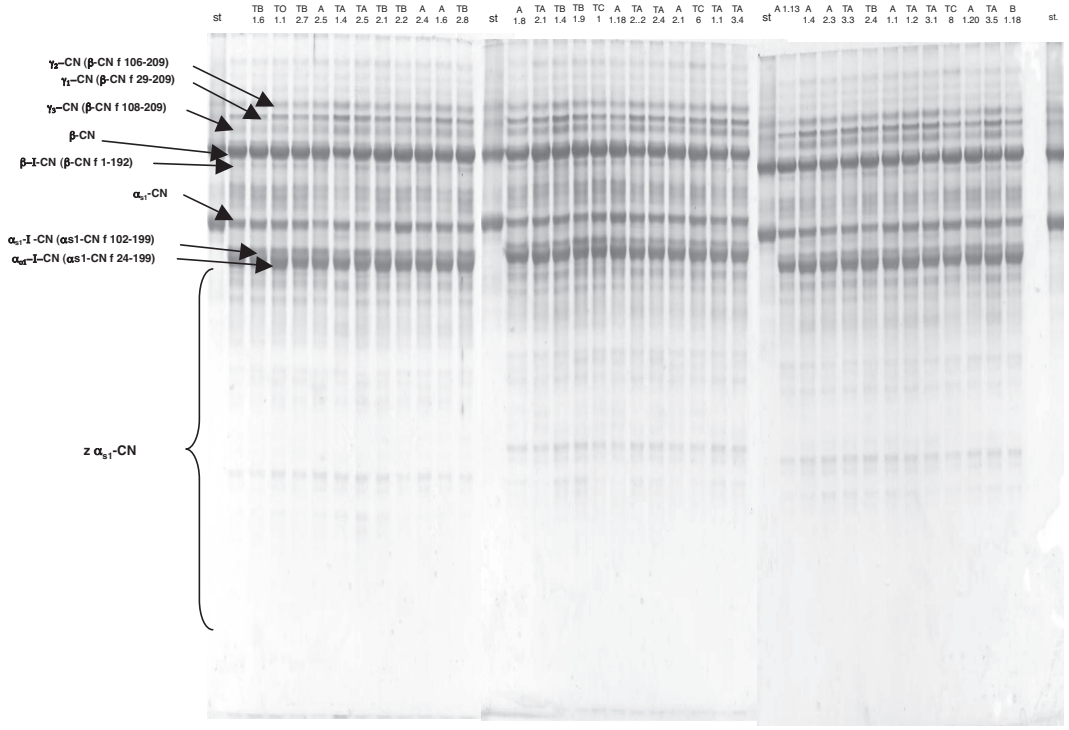


Fig. 2 - Urea-polyacrylamide gel electrophoretograms of miniature Toma Piemontese cheeses made by using different single strains of indigenous bacteria after 60 days of ripening. st = standard Na-caseinate.

that were made by using *Lactobacillus paracasei* TO 1.1, *Lactobacillus fermentum* TA 2.2, *Streptococcus thermophilus* B 1.18, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* TB 2.6 and A 1.20. In all the cheeses, high levels of other peptides were present (marked as z α_{s1}-CN on the electrophoretograms shown in Fig. 2) that had electrophoretic mobilities that were faster than α_{s1}-I-casein. These may have been derived from the action of rennet and indigenous milk proteinases.

The results of the CA of the peak volumes of the bands on the electrophoretograms of the cheeses after 60 days of ripening divided the strains into two groups as shown in the dendrogram in Fig. 3. The first group was made up of 18 *Lactococcus lactis* subsp. *lactis*, 2 *Lactococcus lactis* subsp. *cremoris*, 2 *Streptococcus macedonicus*, 1 *Lactobacillus paracasei* and 1 *Streptococcus thermophilus* strain. The second group was made up of 3 *Lactococcus lactis* subsp. *lactis*, 3 *Streptococcus macedonicus*, 2 *Streptococcus thermophilus*, 1 *Lactobacillus fermentum*, 1 *Lactobacillus paracasei* and 1 *Lactobacillus casei* subsp. *rhamnosus* strain. The cheeses made using strains belonging to the first group were characterised by a greater degradation of the α_{s1}-casein compared to cheeses made using strains from the second group that were characterised by a greater degradation of β-casein.

RP-HPLC

The results of a multivariate statistical analysis of the pH 4.6-soluble fractions of the 30-day-old cheeses, based

on the height of 35 peaks, divided the strains into two groups as shown in the dendrogram in Fig. 4. The first group was composed of 5 *Streptococcus macedonicus* strains, 4 *Lactococcus lactis* subsp. *lactis*, 2 *Streptococcus thermophilus*, 1 *Lactobacillus paracasei*, 1 *Lactobacillus fermentum* and 1 *Lactobacillus casei* subsp. *rhamnosus* strain. The second group was composed of 17 *Lactococcus lactis* subsp. *lactis* strains, 2 *Lactococcus lactis* subsp. *cremoris*, 1 *Streptococcus thermophilus* and 1 *Lactobacillus paracasei* strain. RP-HPLC chromatograms of the pH 4.6-soluble fractions from the 30-day-old miniature Toma Piemontese cheeses made using *Lactococcus lactis* subsp. *lactis* A 1.1 (belonging to the first group) and *Lactococcus lactis* subsp. *lactis* A 1.6 (belonging to the second group) as starter are shown in Fig. 5. The peaks that were recognised and used as variables in the multivariate statistical analysis are indicated. The two groups were characterised by large differences in the heights of peaks 1,6,16,25,26 and 27. The highest peaks of the strains belonging to the first group were peaks 3,4,5,8,10-13,15-18 and 20-29. Most of these peaks were eluted in the chromatogram at retention times from 40 to 55 min and hence were principally large hydrophilic peptides (GONZÁLEZ DEL LLANO *et al.*,1995; PAVIA *et al.*, 2000). The second group, principally made up of *Lactococcus* spp.,

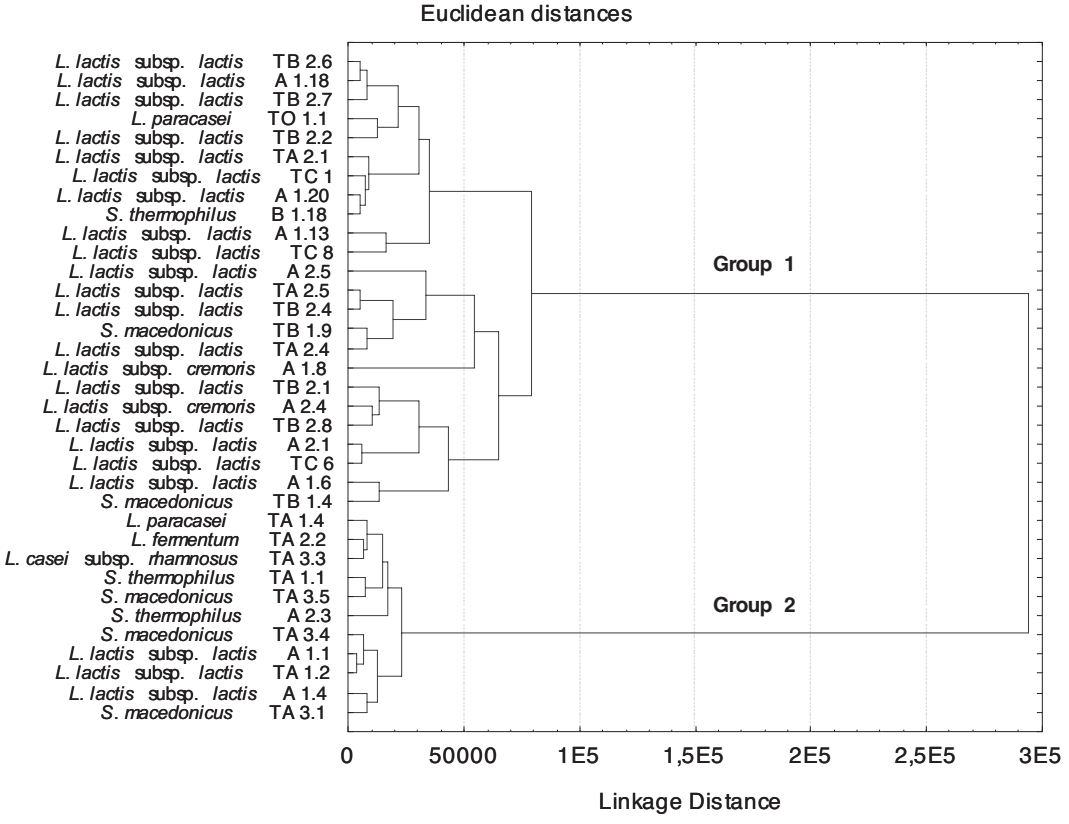


Fig. 3 - Dendrogram from cluster analysis of data obtained from urea-polyacrylamide gel electrophoretograms of pH 4.6-insoluble fractions of the cheeses made by using different single strains of indigenous bacteria after 60 days of ripening.

was characterised by the highest values for peaks 1,2,6,7,9,14,19, and 30-35. These peaks were eluted in the first and last parts of the chromatogram at retention times that were lower than 35 min or higher than 55 min and hence were comprised principally of amino acids and hydrophilic peptides and hydrophobic peptides, respectively (GONZÁLEZ DEL LLANO *et al.*,1995; POVEDA *et al.*, 2003; HAYALOGLU *et al.*, 2004).

The CA of the pH 4.6-soluble fractions of the 60-day-old cheeses, based on the heights of 48 peaks, grouped the strains in the same way as for 30 day-old cheeses except for the *Lactococcus lactis* subsp. *lactis* TB 2.4, *Lactococccus lactis* subsp. *lactis* TA 2.5 and *Streptococcus macedonicus* TB 1.9 strains as shown in the dendrogram in Fig. 6. The RP-HPLC chromatograms of the pH 4.6-soluble fractions from the 60-day-old miniature Toma Piemontese cheeses made using *Lactococcus lactis* subsp. *lactis* A 1.1 (belonging to the first group) and *Lactococcus lactis* subsp. *lactis* A1.6 (belonging to the second group) as starter are shown in Fig. 7. The peaks that were recognised and used as variables in the multivariate statistical analysis are indicated. The two groups were characterised by a notable difference in the heights of peaks 1, 4, 5, 10, 11, 13, 16, 18, 20, 21, 22, 26, 27,29 and 34-40. The strains belonging to the first group were clustered because of the high values of peaks 27 and 37-39 relative to the strains belonging to the second group. Moreover, the

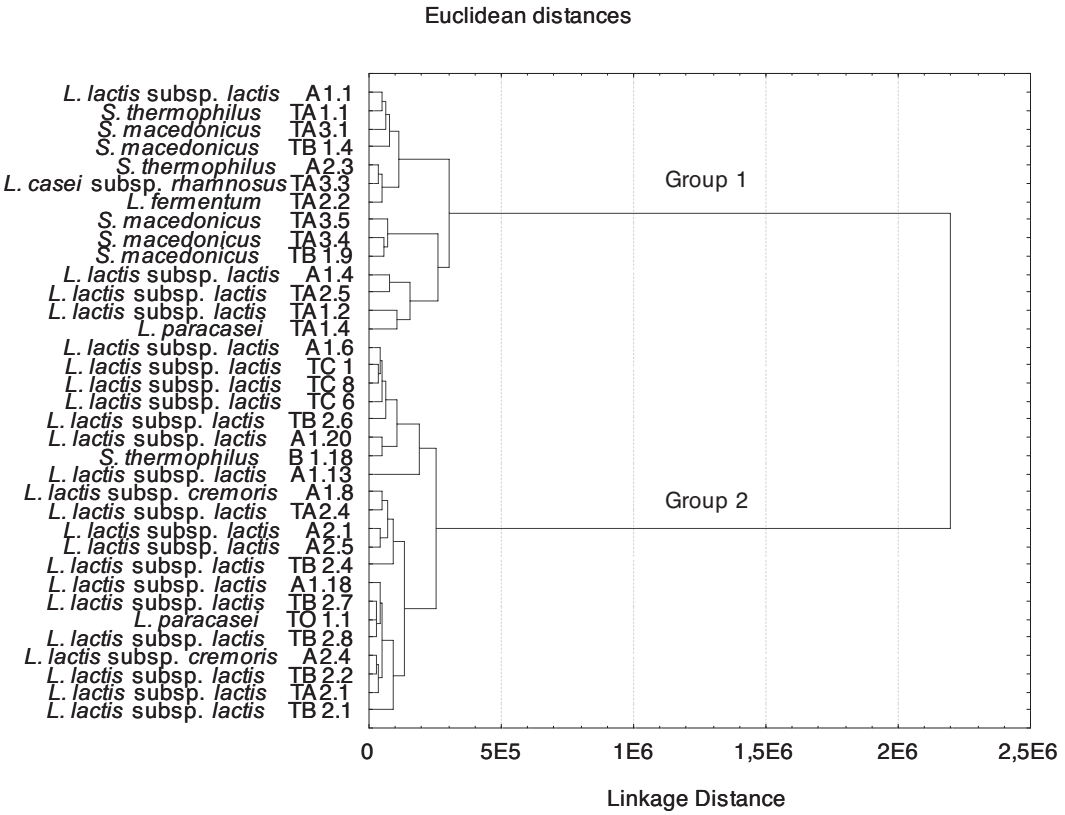


Fig. 4 - Dendrogram from cluster analysis of data obtained from RP-HPLC pH 4.6-soluble fractions of the cheeses made by using different single strains of indigenous bacteria after 30 days of ripening.

strains that formed the second group were clustered because of the high values of peaks 1,4,5 and 13.

Individual free amino acids

Proteolysis in the cheeses was also monitored by determining the levels of individual free amino acids (FAAs) as shown in Fig. 8. The principal FAAs present in 60-day-old cheeses were Leu, Glu, Phe, His and Lys. Cheeses made using the *Lactococcus lactis* subsp. *lactis* strains had the highest concentrations of Leu, Phe, His, Lys, Cys, Met, Ser, Met, Thr and Ile. The cheeses made using *Lactococcus lactis* subsp. *cremoris* strains had the highest concentration of Val

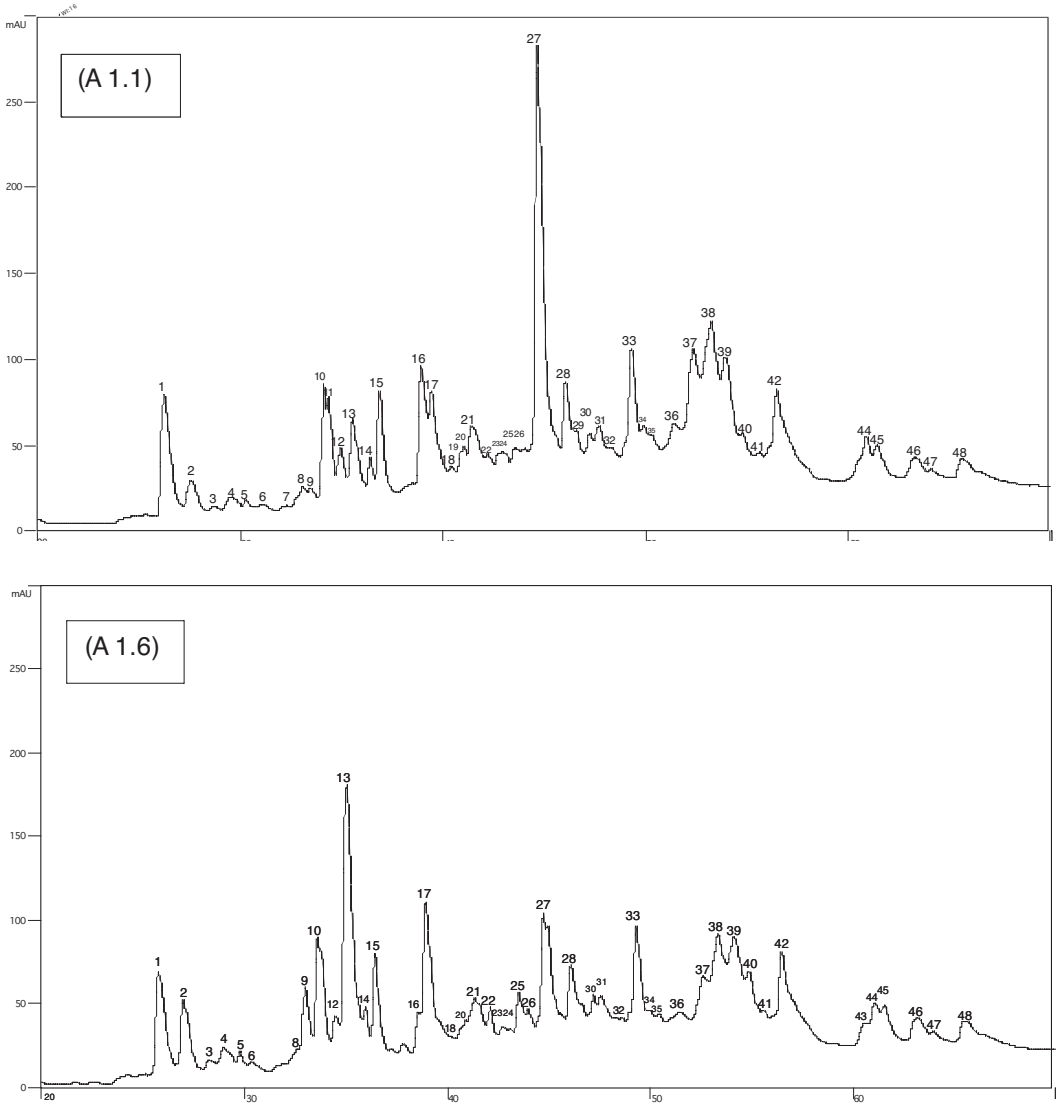


Fig. 5 - RP-HPLC profiles of pH 4.6-soluble fractions of miniature Toma Piemontese cheese made by using the strains *Lactococcus lactis* subsp. *lactis* A 1.1 and A 1.6 after 30 days of ripening.

and Gly and the lowest concentrations of Arg. The cheeses made using *Streptococcus macedonicus* strains had the lowest concentrations of Lys while those made using *Streptococcus thermophilus* strains had the lowest concentrations of Gly. The cheeses made using *Lactobacillus paracasei* strains were characterised by the lowest concentrations of Thr while that made using *Lactobacillus casei* subsp. *rhamnosus* strain was characterised by the highest concentrations of Glu, Asp and Arg and the lowest concentrations of Pro and Met. The cheese made using *Lactobacillus fermentum* strain had the lowest concentrations of Arg, Thr, Ser, Cys, Met, Ile, Leu, Tyr, Try, Phe, His, Arg and Pro. The results of the CA of the FAA data showed that the strains were divided into two groups as shown in the dendrogram in Fig. 9. The first group was made up of 16 *Lactococcus lactis* subsp. *lactis* strains and 1 *Lactococcus lactis* subsp. *cremoris* strain. The second group was made up of 6 *Streptococcus macedonicus* strains, 5 *Lactococcus lactis* subsp. *lactis*, 3 *Streptococcus thermophilus*, 1 *Lactococcus lactis* subsp. *cremoris*, 1 *Lactobacillus casei* subsp. *rhamnosus* and 1 *Lactobacillus fermentum* strain. The two groups were characterised by marked differences in the levels of Leu, Glu, Phe, His, Val, Lys and Pro. The cheeses made using the strains belonged to the second group were characterised by the high-

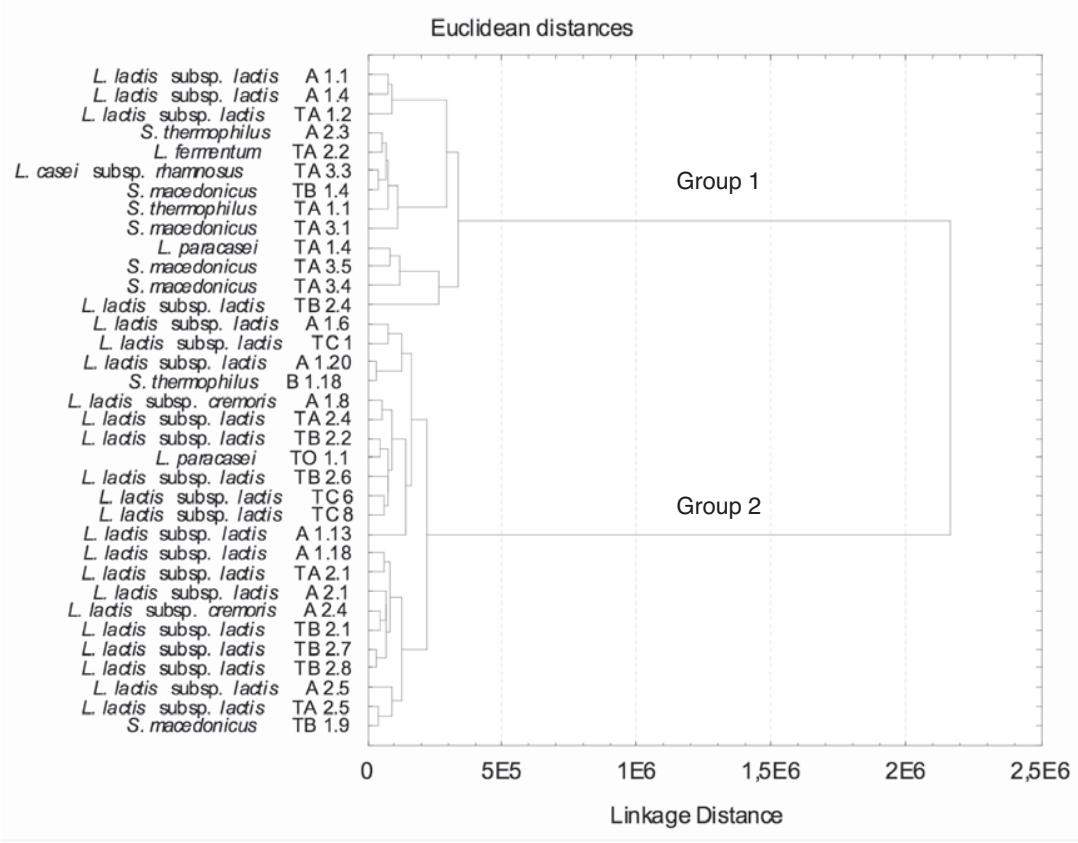


Fig. 6 - Dendrogram from cluster analysis of data obtained from RP-HPLC pH 4.6-soluble fractions of the cheeses made by using different single strains of indigenous bacteria after 60 days of ripening.

est concentrations of all the FAAs except Ala. Starter peptidases are responsible for the production of amino acids (McSWEENEY *et al.*, 1993; LANE and FOX, 1996; CORSETTI *et al.*, 1998). The levels of free amino acids liberated varied due to differences in the enzyme systems of the single strains and the factors that influence their level of autolysis during cheese ripening (WILKINSON *et al.*, 1994a,b; KUNJI *et al.*, 1996; CHRISTENSEN *et al.*, 1999; SAVIJOKI *et al.*, 2006). Other researchers have studied the effect of single strains of lactic acid bacteria on the production of FAAs. O'DONOVAN *et al.* (1996) studied the autolytic properties of three *Lactococcus* strains during cheese ripening. Their research showed that the concentration of FAAs increased in all the cheeses during ripening and that Glu, Leu, and Lys were the major FAAs present at all of the ripening stages. HYNES *et al.* (2001) studied the effect of adding *Lactobacillus plantarum* to different strains of starter bacteria in the production of

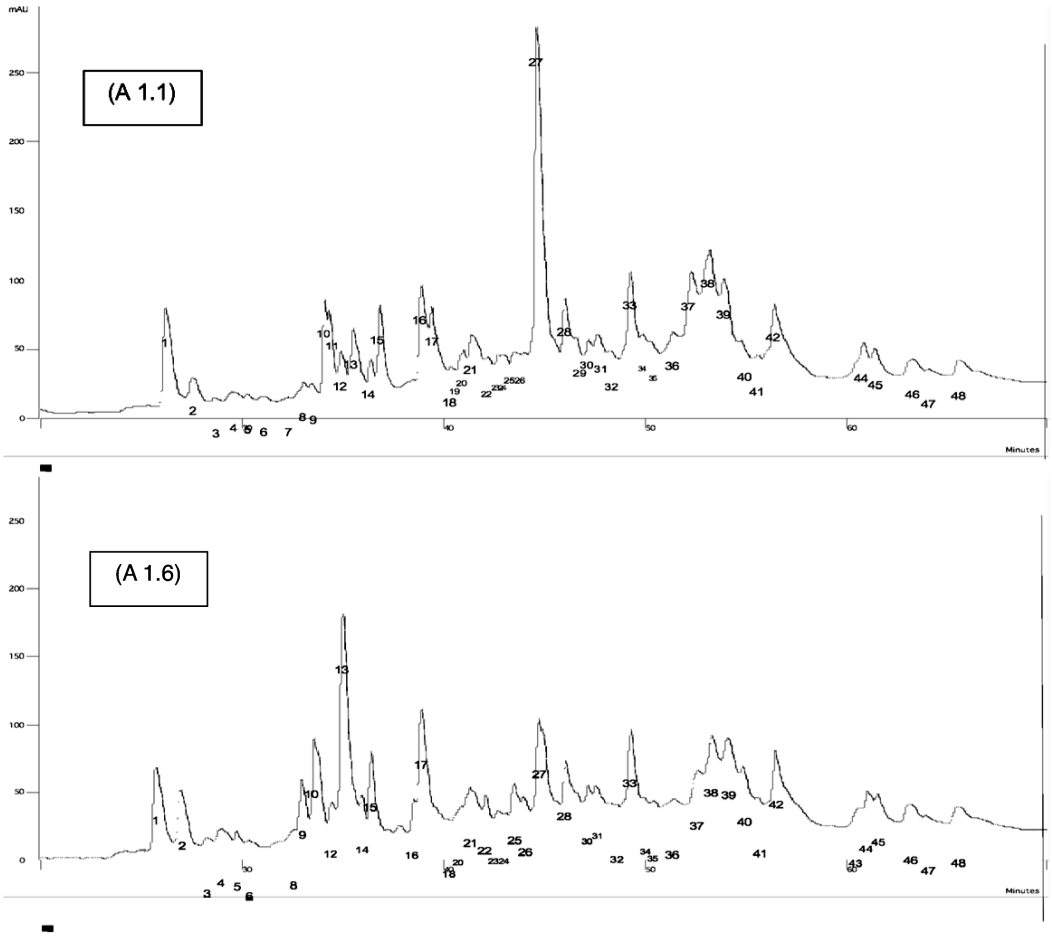


Fig. 7 - RP-HPLC profiles of pH 4.6-soluble fractions of miniature Toma Piemontese cheese made by using the strains *Lactococcus lactis* subsp. *lactis* A 1.1 and A 1.6 after 60 days of ripening.

a washed-curd cheese similar to Saint-Paulin. This study found that Glu, Leu and His were the major FAAs present in cheeses produced with or without the addition of lactobacilli. Cheeses made with the adjunct had higher concentrations of FAA than those made without the adjunct.

MICHAELIDOU *et al.* (2003) studied the effect of a commercial adjunct culture on proteolysis in Feta cheese. The results of their research showed that when strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were added to the normal starter used for cheese production, the level of individual FAAs increased and Leu, Phe and Lys were present in the highest concentrations. HAYALOGLU *et al.* (2004) studied the effect of defined strains of *Lactococcus* on proteolysis in Turkish white-brined cheese and found that Leu, Glu, Phe and Lys were the major FAAs produced by the different strains. POVEDA *et al.* (2004) studied the free amino acid content in Manchego cheese manufactured with different starter cultures and reported that during cheese ripening Glu, Leu, Phe and Lys were present in the highest concentrations. The levels of these FAAs were higher in cheese made with a defined strain-starter or with a defined strain-starter with an adjunct culture than in cheese made using the commercial starter composed of two strains of *Lactococcus lactis*.

CONCLUSION

The use of different strains of indigenous bacteria significantly influenced proteolysis directly and indirectly. The results of the urea-PAGE of the pH 4.6-insoluble fractions of the cheeses were similar at 30 days of ripening but

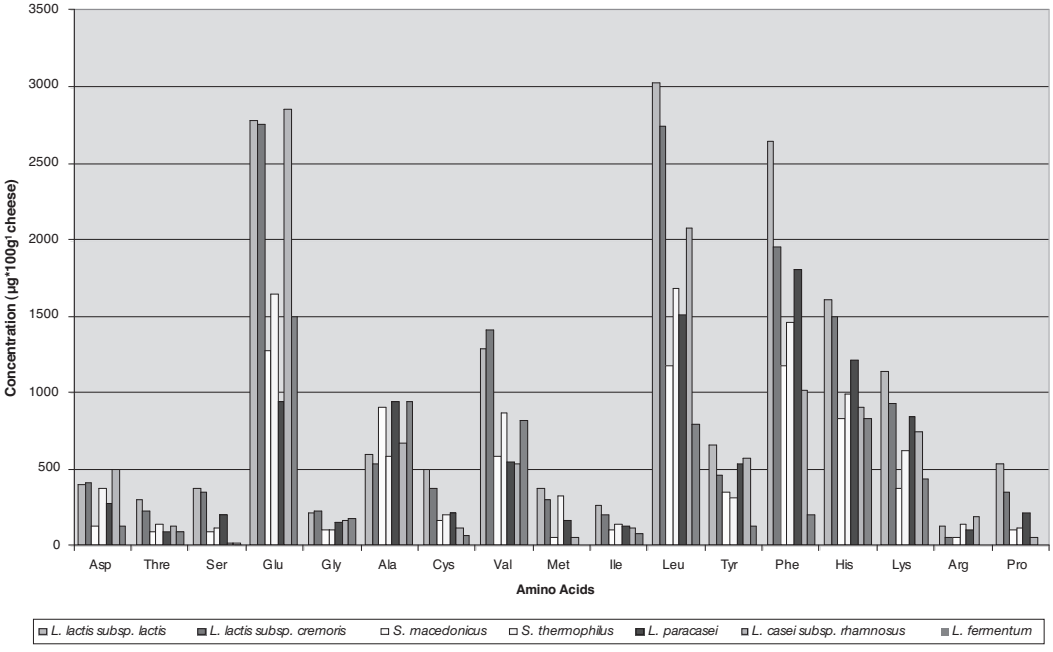


Fig. 8 - Mean concentrations of individual free amino acids after 60 days of ripening in miniature Toma Piemontese cheeses made using different groups of indigenous bacteria as starter.

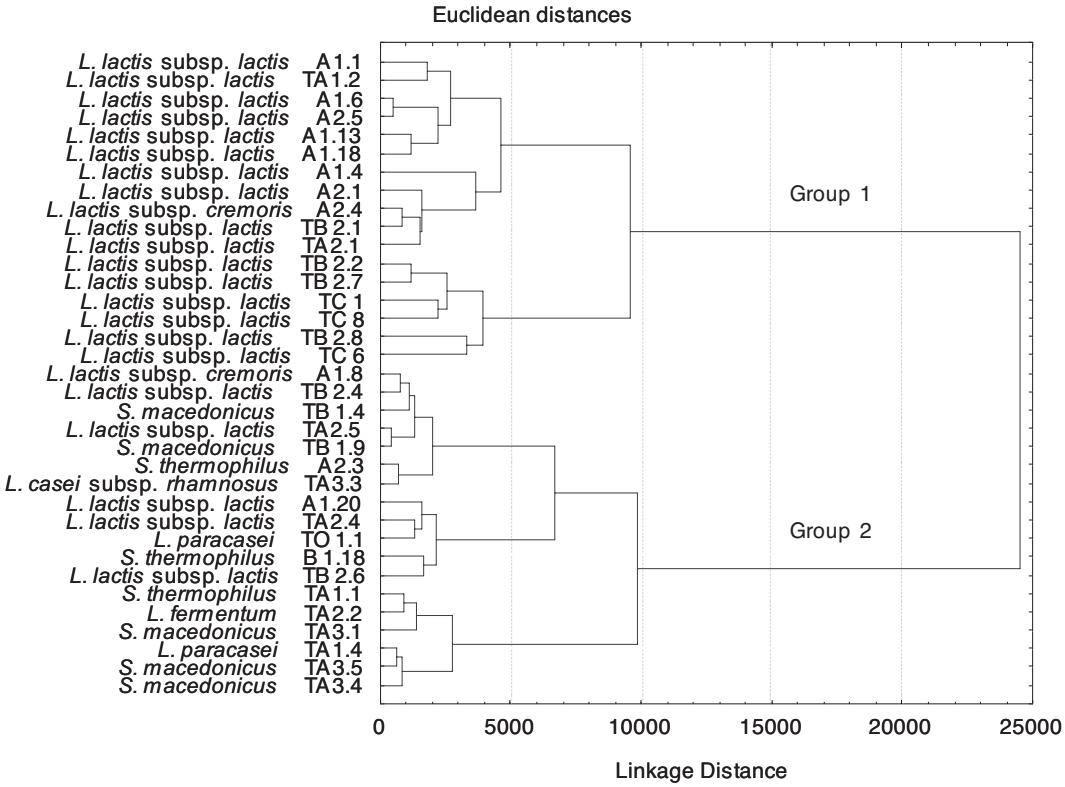


Fig. 9 - Dendrogram from cluster analysis of data obtained from the free amino acid analysis of the cheeses made by using different single strains of lactic acid bacteria after 60 days of ripening.

were very different after 60 days of ripening. Peptide profiles of the pH 4.6-soluble fractions indicated that each strain of indigenous bacteria contributed in a different way to the formation of varying levels of peptides. The different species produced different amounts of the free amino acids. The results of the CA of data obtained after 60 days of ripening, according to the urea-PAGE and the RP-HPLC, showed that the strains were in two groups made up of the same strains except for the strain *Streptococcus macedonicus* TB 1.4.

The data obtained from this study combined with those from other studies could be used to develop new defined-strain starter cultures that could be used in the production of Toma Piemontese cheese.

ACKNOWLEDGEMENTS

M. Bertolino wishes to thank everyone in the Department of Food and Nutritional Sciences, in Cork, for their advice and assistance regarding cheesemaking, urea-PAGE and RP-HPLC analysis.

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Revised paper received March 29, 2007 Accepted June 17, 2007

PAPER

EFFECT OF BAMBOO FIBRE AND GLUCOSE SYRUP AS NEW INGREDIENTS IN THE MANUFACTURE OF *AMARETTI* COOKIES

EFFETTO DELLA FIBRA DI BAMBÙ E DELLO SCIROPPO DI GLUCOSIO QUALI NUOVI INGREDIENTI NELLA PRODUZIONE DEI BISCOTTI *AMARETTI*

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ABSTRACT

"Amaretti", Sardinian almond-based cookies, have a shelf-life of about seven days, due to the quick hardening of the internal moist paste. To improve their overall quality, two different ingredients (bamboo fibre and glucose syrup), known as water-binding agents, were used separately to obtain two different recipes, in addition to the original one. To evaluate their effects, moisture content (wet basis) and a_w were measured. Sorption isotherms were determined and texture (by puncture test) and col-

RIASSUNTO

Gli "Amaretti", dolci sardi a base di pasta di mandorle, possiedono una shelf-life di circa sette giorni, a causa dell'indurimento della pasta interna umida. Per migliorare la loro qualità sono stati utilizzati separatamente due nuovi ingredienti (fibra di bambù e sciroppo di glucosio), ben noti quali agenti in grado di legare l'acqua, ottenendo così due nuove ricette in aggiunta a quella tradizionale. Per valutare il loro effetto sui dolcetti si è deciso di misurare il contenuto umido, l'attività dell'ac-

- Key words: cookies, hardening, humectant, sensory quality, shelf-life, texture, water binding agent -

our (as L^* parameter) evolution were monitored. Moreover, a formal sensorial evaluation was carried out. Moisture content and a_w evolution indicated that both ingredients are effective for preserving the softness of the internal paste for a longer time. After ten days of storage, moisture loss from the internal paste was greater for the traditional cookies (61.2% fall) in comparison with fibre- and glucose syrup-based "Amaretti" (49.3 and 49.7%, respectively). However, sorption data and the puncture test showed that bamboo fibre appears more suitable than glucose syrup for maintaining the typical difference between the external dry crust and the moist almond-based core, controlling water redistribution that takes place in the sugar-dominated formulation. Moreover, colour analysis showed a significant difference between the glucose syrup-based recipe ($L^* \sim 53$) and the other ones ($L^* \sim 75$): this added reducing sugar led to a burnt-like colour of the final product. Finally, the most important difference among the recipes assessed by the descriptive panel test concerned the greater stickiness of the cookies made with glucose syrup, whereas fibre perception was not relevant.

qua (a_w) ed il colore, sono state costruite le isoterme di adsorbimento ed è stata monitorata l'evoluzione della texture nel tempo. Inoltre, è stata condotta una formale valutazione sensoriale dei diversi campioni. L'evoluzione del contenuto umido e dell'attività dell'acqua mostrano come entrambi gli ingredienti siano in grado di preservare per un tempo maggiore la morbidezza interna. In particolare, dopo dieci giorni di stoccaggio, la perdita di umidità dalla pasta interna è stata maggiore per gli "Amaretti" tradizionali (riduzione del 61,2%) rispetto a quelli a base di fibra e sciroppo di glucosio (49,3 e 49,7%, rispettivamente). Tuttavia, la fibra di bambù sembra essere più adatta dello sciroppo di glucosio nel garantire la tipica differenza tra la secca crosta esterna e la morbida pasta interna, come evidenziato dalle isoterme di adsorbimento e dalla analisi tenderometrica. Inoltre, l'analisi colorimetrica ha evidenziato una differenza significativa tra la ricetta a base di sciroppo di glucosio ($L^* \sim 53$) e le altre due ($L^* \sim 75$): l'aggiunta di tale zucchero riducente ha determinato l'imbrunimento del prodotto finale. Infine, la principale differenza tra ricette derivante dal panel test descrittivo ha riguardato la maggiore appiccicosità dei dolcetti ottenuti mediante lo sciroppo di glucosio, mentre la percezione della fibra è stata non rilevante.

INTRODUCTION

In Italy there are many sweets named "Amaretti", but they may differ depending on the region of origin. The Sardinian "Amaretti" are well-known for their typical characteristics that make them unique. Unlike most Italian almond-based cookies, Sardinian ones are dome-shaped, have an intense bitter flavour and a rough, cracked external surface. The most important quality attribute noted by consumers arises from their texture; they have a soft, moist, internal almond-paste, that contrasts with the thin, hard, dry external crust.

According to the definition of the term 'traditional' given by the COMMISSION OF THE EUROPEAN COMMUNITIES (2005) ("Traditional means proven us-

age on the Community market for a period of at least equal to that generally ascribed to a human generation') and by the EuroFIR working group (EUROPEAN FOOD INFORMATION RESOURCE NETWORK, 2005) (that has defined the term traditional as conforming to established practice or specifications prior to the Second World War), "Amaretti" can be cited as traditional regional cookies. Moreover, following the criteria for the definition of a food as traditional reported by TRICHOPOULOU *et al.* (2006), "Amaretti" are manufactured using traditional material, traditional formulation and traditional type of production and/or processing and despite the incorporation of technological progress, they are still produced and processed by traditional methods.

These regional cookies are usually made of sweet and bitter almonds, sucrose and egg white. Moreover, they are characterised by a moisture content (wet basis) of ~ 13-14% for the internal paste and of ~ 5-6% for the external crust; the a_w values are ~ 0.74 and ~ 0.40, respectively. Therefore, the Sardinian Amaretti are good examples of a multi-domain system, having two parts with different moisture content and a_w, in which a continuous moisture migration from one region to another takes place. Multi-domain foods are quite common in all countries, particularly among medium shelf-life bakery products. Among the factors that control the amount and rate of moisture migration in multi-domain foods, the two most important are water activity equilibrium (thermodynamics) and factors affecting the diffusion rate (dynamics of mass transfer). As far as the "Amaretti" are concerned, these factors lead to a very serious problem that affects most cookies (MANLEY, 1988). After a few days (four to seven days), qualitative decay begins due to severe hardening of the internal almond paste that strictly limits the shelf-life of these cookies. Such a short shelf-life prohibits their exportation to countries far away from the traditional places of manufacturing. As reported previously (PIGA *et al.*, 2005; LABUZA, 2001), this hardening could be due to both the redistribution of water (that leads to sugar crystallisation) and loss of water into the surrounding environment. To control this migration, several tools can be utilised to inhibit the change in moisture, including the addition of an edible layer between domains, changing the water activity of the food ingredients, the effective diffusivity of the water, or the viscosity (molecular mobility) in the entrapped amorphous phases (LABUZA and HYMAN, 1998).

Even if the general solutions for obtaining satisfactory multi-domain food items have already been well documented (BELL and LABUZA, 2000; GUILBERT *et al.*, 1996; KROCHTA *et al.*, 1994; LABUZA, 1985; ROCA *et al.*, 2006; THARANATHAN, 2003), to our knowledge there are no studies dealing with the use of different water-binding agents in typical regional cookies, in order to reduce the moisture migration between the different components (crust and paste) and, consequently, the rate of hardening. On the other hand, the need for a standardization of traditional foods, as well as the need to extend shelf life, is increasing, in order to maintain the best quality during a period of time that makes their marketing possible (TRICHOPOULOU *et al.*, 2006). This kind of investigation, dealing with the optimisation and enhancing of traditional food products, has a huge potential for the useful transfer of knowledge and technology in food sectors of great importance in some less-developed regions. Moreover, they can contribute to the transmission of precious aspects of cultural heritage related to particular food products.

The purpose of this research was to test the applicability and effects of glucose syrup and bamboo fibre as water-binding ingredients on "Amaretti" cookies, by a multi-analytical approach. Par-

ticular attention was paid to the changes in the typical sensorial characteristics such as the different textures between the internal paste and the external crust, which is the quality attribute most appreciated by consumers.

MATERIALS AND METHODS

"Amaretti" manufacturing

"Amaretti" cookies were made in the department pilot plant, using traditional ingredients in the following amounts: sweet almonds (650 g); sucrose (650 g); fresh liquid egg white (255 g); bitter almond aroma (5 mL).

The sweet almonds (purchased in a local market) were ground into tiny pieces with a mincing machine (SIMAC, Bravo chef 750, Veldhoven - Holland). Two sieves (Analysensieb - RETSCH, D-42759 Haan - Germany) both certified in conformity with DIN-ISO 3310/1 standard, were used to obtain two different sized particles, the first with a diameter < 1.0 mm (350 g) and the second with a diameter of 3.55 and 2.36 mm (300 g), respectively. Both fractions were put in a small bowl and all the sugar and most of the egg white (165 g) were added. The ingredients, left unmixed, were placed in a climatic chamber (Heraeus Vötsch, mod. HC 0020 - Balingen - Germany) at 15±0.5°C and 40±2% RH for 24 h. This pre-mixture was then put in a refiner machine (Steno, f.lli Nazzari, mod. R2 - Italy) to obtain a more homogeneous matrix, which was subsequently mixed for 3 min at low speed with a vertical axis planetary mixer (Hobart Corporation, mod N-50, Troy, OH 45374) with a stainless steel paddle. The remaining part of the egg white (90 g) and the bitter almond aroma were added at this point. The ingredients were then mixed for 5 min at medium speed. The mixture was then used to form the cookies on a baking pan. To shape the cookies as uniform as possible, small steel discs of about 5.5 cm in diameter were used, the paste was put into the forms (40.0±0.5 g) by using a typical confectionery cloth-bag with a stainless steel tip of 1.3 cm in diameter. Baking was carried out in a ventilated electric oven (Moretti Forni s.r.l., mod. Mikro - Italy) for 20 min at 200°C. Finally, 38 "Amaretti" cookies (37±0.5 g) were obtained (Fig. 1), which were then cooled in a climatic chamber at 25±0.5°C and 40±2% RH for two h.

Ingredients

To test the effects of the two different water binding agents, the "Amaretti" were also produced adding 30 g (~2% $^{w}/_{w}$) of glucose syrup (D.E. = 47), provided by a local confectioner's shop, and 30 g (~2% $^{w}/_{w}$) of bamboo fibre (Chimab S.p.a., Campodarsego (PD) - Italy) before starting the mixing. The amounts of these ingredients were selected for two reasons: first, larger amounts of ingredient would affect food-processing, leading to a paste that would be hard to handle particularly during mechanical

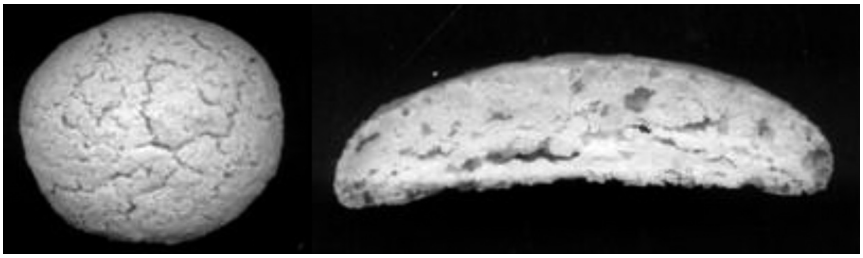


Fig. 1 - Section of a typical "Amaretti" cookie (left) and its section (right).

mixing. Second, the above-mentioned amount should not affect the original quality characteristics of these regional cookies and hence their "traditional" attribute. Glucose syrup was chosen as the conventional sugar used in the confectionery industry (GUILBERT, 2002), and is characterised by low cost, ready availability on the internal market and, from a legal point of view (DIRECTIVES 2000/13/EC and 2003/89/EC), it is considered a food ingredient. In this work, the glucose syrup used had an average D.E. value (D.E. = 47). The viscosity of the syrup is a parameter that influences the dynamics of mass transfer by reducing the moisture migration between the internal paste, crust and surrounding environment. In fact, the rate of water diffusion is controlled by the local viscosity of the system. The higher the local viscosity, the less molecular movement is noted with respect to rubbery and glassy states (LABUZA and HYMAN, 1998). Moreover, glucose syrup is recognised as a humectant agent thanks to its colligative properties (BELL and LABUZA, 2000). Bamboo fibre is a new cellulose-based ingredient derived from the shoots of bamboo plants. This is a purified fibre (total fibre content >99% on dry basis). In addition, it is characterised by a high pectin and hemicellulose content. It is about 0.25 mm long, has a density of about 120 g/L and a *WHC* (water holding capacity) of 8.7 g/g fibre, calculated after centrifugation. With these characteristics, the fibre can retain water, not only by capillary forces, but also by specific bonds (data provided by the supplier). For these reasons, the addition of this fibre enables the moisture loss to be reduced considerably; it can also improve textural properties, since it is also considered to be a texturising agent. Moreover, the fibre has zero calories, is inert, tasteless and white in colour. Its functional properties are no less important; more than 90% of the water-insoluble fibre bulks in the stomach, removes undesired metabolic by-products and shortens intestinal transit time. Dietary fibre is essential for keeping the digestive tract healthy and has also been related to a variety of beneficial effects, such as contributing to weight management (RODRIGUEZ *et al.*, 2006). Due to consumer interest in health foods and beverages, fibre-fortified foodstuffs is a category with potential for fast-growth. Another important aspect is that any plant fibre, including that from bamboo can be labelled simply as plant fibre under the listing of food ingredients and not as an additive (as noted above for glucose syrup).

From now on, the three different typologies of "Amaretti" will be indicated as follows: *T*= traditional; *S*= syrup; *F*= fibre.

Storage conditions of "Amaretti" cookies

For each trial the "Amaretti" were divided into three lots: one for the determination of the sorption isotherms, one for textural analysis and the last for the evaluation of the a_w, moisture content (wet basis) and colour. The cookies were then stored under controlled temperature-humidity conditions (25±0.5°C and 40±2% RH, similar to those of the typical outlets for "Amaretti"), for ten days and analysed after 1, 2, 4, 6, and 10 days. This storage time was selected to verify the single effect of the added ingredients on "not-packaged Amaretti" after a period of time slightly longer than the typical shelf-life of "packaged Amaretti".

Water activity and moisture content

Water activity was measured on three "Amaretti" at a time with an electronic hygrometer (Aqua Lab Series 3 mod. TE – DECAGON DEVICES, Inc. Pullman, Washington USA), previously calibrated with a standard solution of NaCl of

known activity (prepared by High-Purity Standards for DECAGON DEVICES, Inc.). Gravimetric analysis was performed in triplicate to determine water content (% H_2O on wet basis) using an oven at 130±2.0°C for 90 min, long enough to guarantee the total drying of the samples. The measurements were performed separately on the whole cookie (paste and crust) and on the internal paste. In the first case, 5±0.5 g of "Amaretti" were cored with a spoon soil auger, whereas in the second case, 5±0.5 g were sampled from the inner part of the almond paste filling with a laboratory spattle.

Moisture sorption data

Moisture sorption data were collected according to the procedures reported in the COST project of European Cooperation in the field of Technical and Scientific Research (WOLF *et al.*, 1985) and the principles described by BELL and LABUZA (2000). In particular, whole "Amaretti" were used because it was impossible to separate in a proper way the external crust from the internal paste. Even though the term "sorption isotherm" is here used, the more precise term is "working isotherm", i.e. a combined adsorption/desorption isotherm (LABUZA, 1985). Triplicate samples were placed in sealed glass jars with an internal inert platform used to raise the samples off the floor. Each jar containing saturated salt slurries in the a_w range of 0.11 to 0.81 were stored in a climatic chamber at 25°C. At high water activities (a_w > 0.75), a small amount of toluene (1 g) was placed in a capillary tube fixed in the jar to prevent microbial spoilage. Salts (LiCl anhydrous, CH_3COOK, $MgCl_2$ hexahydrate, K_2CO_3 anhydrous, $Mg(NO_3)_2$ hexahydrate, CH_3COOLi dihydrate, NaCl, $(NH_4)_2SO_4$) used to obtain constant water activity environments were reagent grade (Sigma Aldrich Srl, Via Gallarate 154, 20151 Milano). For all samples, equilibrium was reached in three weeks.

Texture analysis

Hardness was evaluated in cooled, freshly baked "Amaretti" and after 2, 4, 6 and 10 days by using a food texture analyser (mod. Z005, Zwick Roell, Ulm, Germany). The software "TestXpert V10.11 Master" was used for the data analysis. Textural determination was made on 15 "Amaretti" per each storage time by using a 4 mm diameter cylinder probe for a puncturing test (BOURNE, 2002). "Amaretti" were punctured in the center up to 9 mm in depth, in order to check whether any different structural characteristics were present inside or on the surface. These analyses were carried out by using two different cell loads, depending on the time of analysis: a 100 N cell load for the first three trials (after 0, 1, and 2 days of storage) and a 5 kN cell for the last three (4,6 and 10 days). The parameters for the puncturing test were: pre-test speed: 100 mm min^{-1}; pre-load: 0.01 N (with a 100 N cell load) and 2 N (with a 5 kN cell load); pre-load speed: 4 mm min^{-1}; test speed: 10 mm min^{-1}; post-test speed: 700 mm min^{-1}; distance: 15 mm; probe: 4 mm diameter cylinder. "Amaretti" were selected with a diameter of 55±2 mm and a thickness of 25±1 mm, in order to have standardised samples. Hardness measurements of samples by puncturing involved plotting force (N) versus deformation (mm) and calculation of three parameters: (a) maximum force (N) as an index of the hardness of the crust (F_{max}); (b) elastic modulus (Nmm^{-1}) as an index of the stiffness of the crust (E_{mod}); (c) area under the curve (Nmm) up to 9 mm as an index of global hardness of the cookie (W_{tot}).

Colour measurement

The external colour of "Amaretti" was measured using a reflection colorimeter

(MINOLTA Chroma Meter mod. CR 210, Osaka, Japan). Cookies were placed on a white standard plate (L^* = 100) and the *CIE L*a*b** coordinates were measured, using D65 illuminant/10° observer. Colour analysis was carried out only on freshly baked "Amaretti" (30 replicates for each lot).

Sensory evaluation

A sensory evaluation was carried out to check the texture evolution during storage time and the difference between recipes. In particular, a descriptive test was used in which nine trained panellists (5F, 4M, age range 21-58 yrs) described the evolution of the cookies through four attributes: resilience, external crispness, internal stickiness and fibre perception. For each of the five times of analysis (freshly baked "Amaretti" and after 2, 4, 6 and 10 days of storage), the sensory test was carried out using six "Amaretti" (two for each formulation), presented to the panel in two separate sessions. An unambiguous alphanumeric code was given to each recipe, to avoid that the panellists underwent any kind of possible influence. To best assess the sensations from the products, this chronological order was followed: first a general look at the product, then touch (resilience), then take the first bite (external crispness) and then further chew the cookie (internal crispness and fibre perception). The panellists attributed a value between 1 (minimum) and 9 (maximum) for each of these parameters.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using Statgraphics Plus 4.0 software. In particular, the data were grouped by lots (the three different recipes: T, S and F) to assess the differences for each recipe. The mean values, when appropriate, were separated by LSD's multiple range test at P≤0.05.

RESULTS AND DISCUSSION

Moisture content and water activity

The two added ingredients gave rise to different moisture content and a_w values of the internal paste (Table 1). Cookies with syrup had a higher moisture content, in comparison to the other two recipes, due to its composition (water/sugar solution), whereas the a_w value of the internal paste of S freshly baked "Amaretti" was not statistically different from the a_w value of the traditional cookies, in contrast to cookies with fibre, that had the lowest a_w value. Although fibres are not classified as humectants

Table 1 - Moisture content (%) and a_w values of the three different recipes of freshly baked "Amaretti".

	Traditional		Syrup		Fibre	
	Paste	Paste + crust	Paste	Paste + crust	Paste	Paste + crust
Moisture content	12.82^a (±1.36)	7.56^c (±1.09)	15.61^b (±1.18)	8.81^c (±1.45)	12.90^a (±0.94)	8.22^c (±1.62)
a_w	0.74^d (±0.025)	0.57^f (±0.011)	0.72^d (±0.013)	0.55^f (±0.020)	0.70^e (±0.025)	0.54^f (±0.018)

Standard deviation is reported in brackets.
Different letters denote statistically significant differences (P≤0.05) among recipes for each moisture content and a_w value (internal paste and paste + crust).

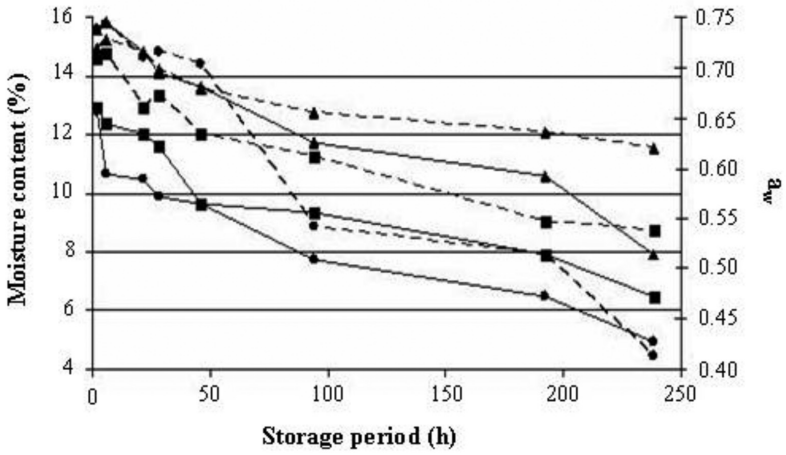


Fig. 2 - Change of moisture content (%) and a_w of paste in the three different "Amaretti" recipes. Data are the means of three determinations.
Legend: - - - - - a_w _____ moisture content (%) ● T ▲ S ■ F

because they do not lower water activity, nevertheless this result could somehow be related to their great capacity to hold water. All the pastes had a water activity around 0.72±0.02. With regard to the evolution of the above-mentioned parameters (Fig. 2), on the last day of storage the loss of moisture from the internal paste was particularly evident in T (61.2% decrease) and less pronounced in S and F (49.3% and 49.7%, respectively). The same behaviour was observed for the a_w values. In fact, all of the types of "Amaretti" underwent a progressive decrease in a_w, but it was sharper in T than in the other two. In the case of fibre, this trend could again be linked to the *WHC*. Regarding glucose syrup, the delayed moisture loss is linked to its colligative properties and to the increased internal viscosity of the system.

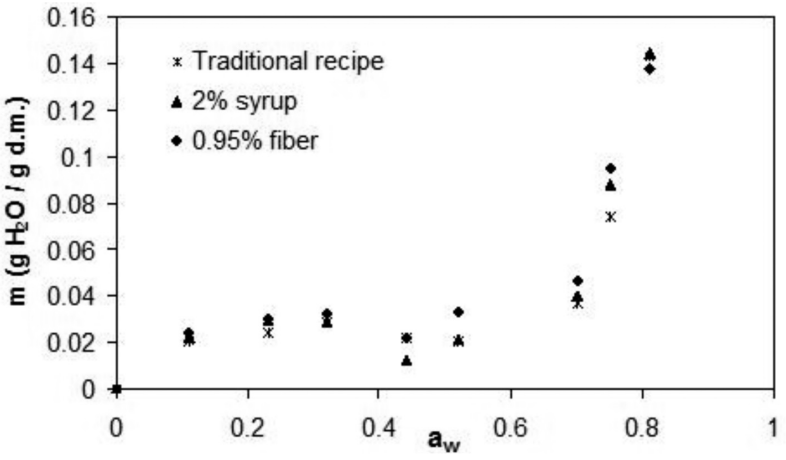


Fig. 3 - Experimental sorption isotherm curves for the three different recipes.

Moisture sorption data

Moisture sorption data were obtained to assess the different effects of the two new ingredients on the whole cookie (BELL and LABUZA, 2000). Fig. 3 shows the sorption isotherm data obtained from the three different recipes of freshly baked "Amaretti" after an equilibrium time of 3 weeks at 25°C. Each data point is the average of three samples (Table 2).

The shape of the isotherms is different from a typical sigmoid curve. In fact, the latter is a smooth curve in the 0.30-0.52 a_w range, while the three experimental isotherms exhibit a slight discontinuity, probably due to sucrose crystallisation, as reported in previous papers (PIGA *et al.*, 2005; SALTMARCH and LABUZA, 1980) but further study is needed to confirm this hypothesis (e.g., MRI). The imperfect coincidence of the curves is quite evident, especially for some a_w values; there is no statistically significant difference among the first three a_w values (i.e., 11, 23 and 32% relative humidity equilibrium, ERH). The three different lots of "Amaretti" lost the same amount of water. This moisture loss at the lowest a_w levels was assumed to be due to desorption. At 0.44 a_w there was a statistically significant difference between S on the one hand and T and F on the other. As noted above, sugar crystallization probably took place more rapidly in S than in the other two recipes. Generally, a sugar mixture is expected to prevent this phenomenon but in this sugar-dominated formulation, both saccharose and glucose are in an amorphous form (highly hygroscopic), which promotes water redistribution at the molecular level (e.g., water in different regions such as sugar and protein). At 52% ERH, a significant difference was observed between S and T on the one hand and F on the other, since at this a_w value the effect of crystallisation was less evident. The water mobility (and consequent water redistribution between components) was less due to the high water binding capacity of the fibre. At 70% ERH, there was a significant difference between T and F, whereas at a_w value of 0.75 there was a difference between T on the one hand and S and F on the other. This result reflects the water-binding affinity of the different matrices. At the highest a_w value there were no statistically significant differences between lots; this is because the "Amaretti" took moisture from the surrounding air very easily, when the humidity was high. This moisture gain at the highest a_w levels was assumed to be due to sorption. Based on this, the discontinuities exhibited by the isotherms would be the result of the loss of moisture due to sugar crystallisation. At high a_w values, i.e., ~0.70 and above, there would be losses of moisture over time during the equilibration period as a re-

Table 2 - Sorption isotherm data obtained for the three different recipes.

Recipe/a_w	0.11	0.23	0.32	0.44	0.52	0.70	0.75	0.81
Traditional	0.020493^a (±0.00387)	0.024408^a (±0.00099)	0.029104^a (±0.00348)	0.022151^a (±0.00479)	0.020516^a (±0.00159)	0.036925^a (±0.00564)	0.074292^a (±0.00280)	0.142684^a (±0.00646)
Syrup	0.022206^a (±0.00636)	0.02942^a (±0.00925)	0.029039^a (± 0.00291)	0.012856^b (± 0.00303)	0.021116^a (±0.00574)	0.040126^{ab} (±0.00303)	0.087975^b (±0.00795)	0.144599^a (±0.00795)
Fibre	0.024278^a (±0.00452)	0.030402^a (±0.00579)	0.032457^a (±0.00290)	0.022109^a (±0.00116)	0.032976^b (±0.00484)	0.046681^b (±0.00518)	0.094931^b (±0.00331)	0.137724^a (±0.01639)

Standard deviation is reported in brackets.
Different letters indicate statistically significant differences ($P \leq 0.05$) among recipes for each a_w value.

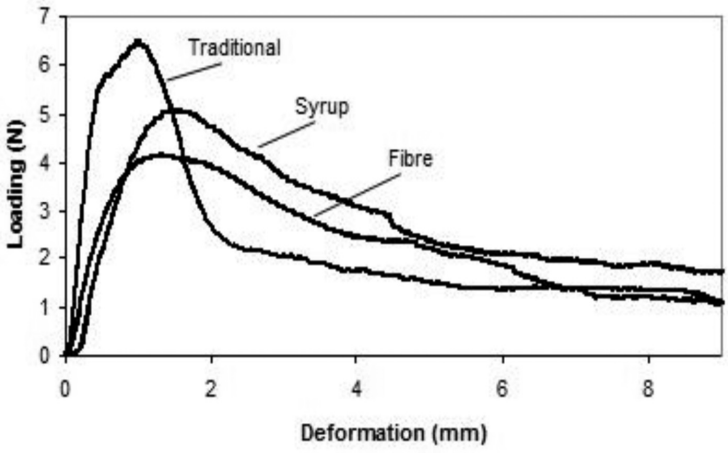


Fig. 4 - Three mean curves of freshly baked "Amaretti".

sult of continued sugar crystal growth, but this would not be reflected in the isotherms which would show a smooth curve with increasing a_w because the different components within the matrix (protein) can hold more water (SALTMARCH and LABUZA, 1980). In this case, moisture uptake exceeds moisture loss from sugar crystallisation.

Texture evolution

The curve resulting from the puncturing test on freshly baked "Amaretti" is characterised by an initial increase, a maximum point and a final decrease that corresponds, respectively, to an increasing force recorded at the onset of penetration, the maximum force record-

Table 3 - Mean values of the three parameters arising from texture analysis of freshly baked and after storage "Amaretti".

Days of aging	E_{mod} (Nmm^{-1})			F_{max} (N)			W_{tot} (Nmm)		
	Traditional X (±s.d.)	Syrup X (±s.d.)	Fibre X (±s.d.)	Traditional X (±s.d.)	Syrup X (±s.d.)	Fibre X (±s.d.)	Traditional X (±s.d.)	Syrup X (±s.d.)	Fibre X (±s.d.)
0	21.54^{a} (±6.35)	7.85^{fn} (±1.15)	8.98^{in} (±2.89)	6.01^{a} (±0.70)	5.18^{f} (±0.35)	4.49^{j} (±0.28)	17.18^{an} (±2.88)	21.60^{d} (±3.34)	18.41^{gn} (±2.60)
2	97.96^{abo} (±46.18)	4.94^{f} (±2.01)	122.74^{io} (±21.10)	42.90^{b} (±11.07)	4.20^{f} (±0.52)	25.46^{l} (±2.34)	94.74^{a} (±19.13)	26.32^{d} (±3.49)	62.19^{h} (±10.87)
4	202.83^{bc} (±151.61)	8.42^{fp} (±2.34)	33.22^{lp} (±8.72)	80.19^{c} (±10.76)	5.22^{f} (±1.14)	25.12^{l} (±3.32)	445.07^{b} (±91.01)	32.75^{d} (±5.08)	132.05^{i} (±22.34)
6	283.10^{cd} (±160.14)	33.22^{gq} (±6.65)	226.28^{lq} (±28.83)	121.01^{d} (±20.06)	22.29^{g} (±2.60)	86.16^{m} (±6.03)	666.48^{c} (±133.00)	98.05^{e} (±14.80)	286.58^{l} (±47.91)
10	368.47^{de} (±207.33)	90.32^{hr} (±51.75)	136.45^{mr} (±76.73)	139.89^{e} (±28.88)	39.82^{h} (±11.10)	100.27^{n} (±8.33)	606.53^{c} (±157.8)	131.86^{f} (±36.03)	499.09^{m} (±68.09)

Standard deviation is reported in brackets.
Different letters indicate statistically significant differences ($P \leq 0.05$) within formulation and among recipes for each texture index.

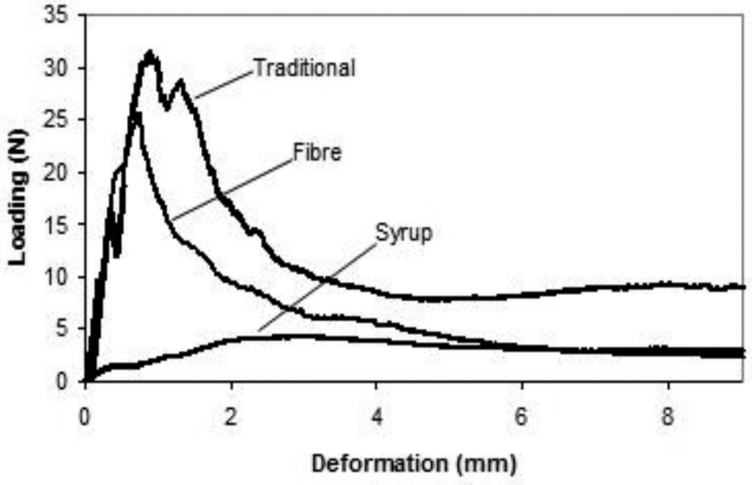


Fig. 5 - Three mean curves of "Amaretti" after 2 days of storage.

ed when the probe penetrated the cookie and the penetration of the internal paste. This shape is typical for food systems characterized by an outer surface (the external crust, in this case) that is harder than the remaining part (the internal paste). The profiles of the three mean curves of freshly baked "Amaretti" is similar (Fig. 4); T had an external crust that was little harder and stiffer than the other two, as can be seen from the maximum force and Young's modulus values, respectively (Table 3). The three curves are almost coincident in the final tract, indicating that the internal paste had the same, if not equal consistency, even if not equal. This was confirmed by the overall hardness (see the area under the curves and the corresponding W_{tot} values in Table 3). There was a significant difference between S "Amaretti" on the one hand and T and F on the other. This was due to the viscosity of the internal paste, which gave a slightly upwards shifted curve. After 2 days of storage (Fig. 5), the curve related to S was flatter than that of the previous day; moreover, the peak due to the crust penetration was absent. This could be due to the water redistribution between components within the matrix, i.e. the water moves to the hygroscopic sugar in the amorphous form and from the paste to the crust, due to the a_w gradient. Finally, the crust lost its crispness as can be seen from the maximum force and Young's modulus values (Table 3). The F curve was similar to that of S in the final part, but very different in the first; in fact, the F "Amaretti" had a hard, crumbly crust that was still differentiated from the internal soft paste. The T cookies had a crust that was as stiff as F (no significant difference concerning Young's modulus), but harder (higher maximum force). Moreover, it is interesting to observe that the descending tract of the T curve was slightly shifted over the other two, indicating that after two days, the internal paste of T had already lost a little more moisture. The above-described trend becomes more evident after 4 days (Fig. 6). T "Amaretti" underwent a severe hardening; the area under the curve as well as the maximum force increased dramatically (Table 3) and there was no difference between the texture of the paste and that of the crust. The curve of the S cookies had a flat profile, indicating that the applied force was the same throughout the whole thickness of the "Amaretti". This means that the moisture redis-

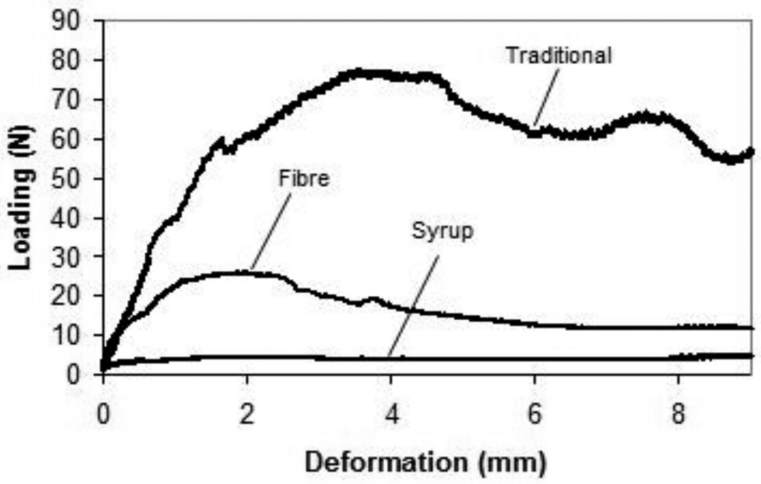


Fig. 6 - Three mean curves of "Amaretti" after 4 days of storage.

tribution continued, leading to a structural change of the crust. In the cookies with added fibre, there was only a slight hardening of the internal paste. On the sixth day of analysis (Fig. 7) the hardening also began to affect S; the moisture loss exceeded the moisture redistribution between the components. The original difference in texture between paste and crust was observed in F, where the effect of the fibre was very noticeable (it should be noted that the water binding capacity of the fibre used in this work was 8.7 g/g fibre). However, these "Amaretti" were harder than those analysed in the previous time period as can be deduced by observing the value of the area under the curve (Table 3). On the last day (Fig. 8), it is interesting to observe that only the T "Amaretti" broke into pieces during the test, due to the porous structure previously occupied by

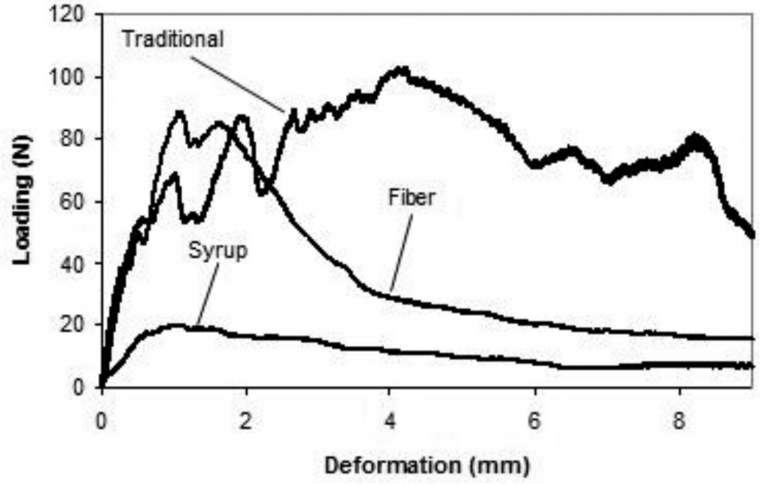


Fig. 7 - Three mean curves of "Amaretti" after 6 days of storage.

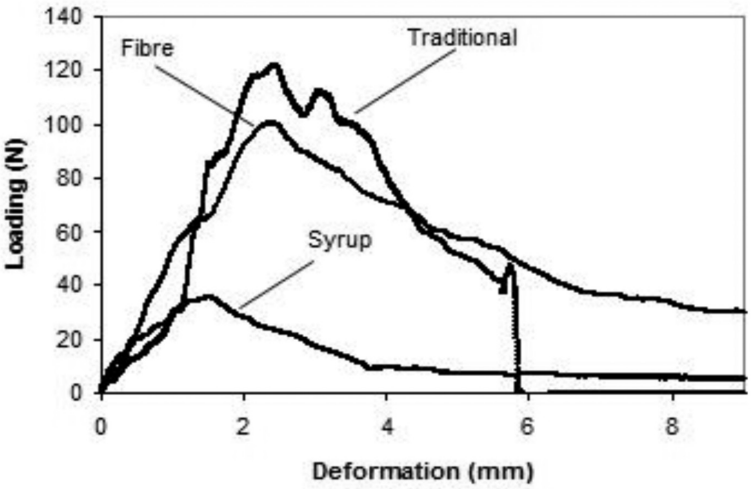


Fig. 8 - Three mean curves of "Amaretti" after 10 days of storage. The "breaking-point" for the traditional formulation is well evident.

water. In contrast, the other two recipes did not break apart because water was still present in the matrix. The different textures between paste and crust were still evident in both S and F. The overall hardness was higher in F (value of maximum force under the curve), whereas the crust of S was stiff but not as hard as can be seen from the maximum force values (Table 3).

Colour measurement

The results of the colour analysis are reported in Table 4. Regarding the *CIE L*a*b** parameters for S "Amaretti", the *a** parameter value was the highest indicating a hue of redness/brownness. At the same time they had the lowest *L** value, indicating a greater overall darkness of these cookies. This was due to the Maillard reaction, that proceeded more intensely in S due to a higher amount of reducing sugar. The values of the *CIE L*a*b** parameters for F exhibited the highest *L** value and the lowest *a** and *b** values, indicating an overall lighter colour and less brownness in the cookies.

Sensory evaluation

The panellists who took part in the sensory evaluation had the task of detecting any changes in the texture that occurred in the "Amaretti" during the storage time (ten days). In particular,

Table 4 - *CIE L*a*b** parameters for the three different recipes.

CIE parameters	Traditional	Syrup	Fibre
*L**	+73.72 (±1.30)^{c}	+53.08 (±2.32)^{d}	+78.35 (±0.7)^{e}
*a**	+5.05 (±0.13)^{f}	+15.54 (±0.50)^{g}	+2.79 (±0.01)^{h}
*b**	+27.28 (±0.58)^{i}	+26.85 (±2.29)^{i}	+23.49 (±0.3)^{l}

Standard deviation is reported in brackets.
Different letters indicate statistically significant differences ($P \leq 0.05$) among recipes for each *CIE L*a*b** parameters.

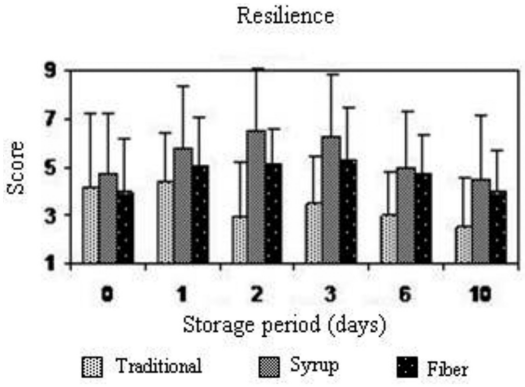


Fig. 9 - Sensorial analysis: resilience evolution during 10 days of storage.

four parameters that can be well described by the human senses were taken into consideration. The parameters correlated to the senses of odour and flavour were not taken into account, but only those related to the texture evaluation. Resilience (Fig. 9) was considered as the tendency of the crust to return to its original shape after the removal of a stress (the slight pressure of the index finger in this case) that produced elastic strain. In this case, the panellists confirmed the results previously obtained with the puncture test. In the first few days after baking, this attribute was perceived more clearly in the S "Amaretti" than the F ones. The former underwent a water redistribution between the paste and crust that led to a soggy, elastic crust; the panellists perceived this sensation up to the sixth day of storage, after which there was moisture loss from the external crust to the surroundings. In T, a low resilience was perceived in the first days, which disappeared after three days due to a quick hardening of the crust. As regards the external crispness (Fig. 10), the lowest scores were given to S because the crust began to pick up water, became less crisp (particularly evident on the third day of analysis). This led to quality decay of the "Amaretti", in which crispiness is an essential characteristic that influences consumer acceptance. This parameter was perceived later in T and F, where the moisture redistribution was less intense than in S. On the last day of analysis, the external crispiness scores were lower than the initial days for all three lots, because of the hardening of the crust due to the loss of moisture into the surrounding environment. With regard to internal stickiness (Fig. 11), the panellists perceived S to be stickier than the other two lots. This was certainly due to the addition of glucose syrup; its thickness was perceived as sticky by the panellists. While this ingredient reduced moisture migration from the internal paste, it also affected the original texture of the final product. With regard to fibre perception (Fig. 12), the panellists were not able to distinguish the presence of fibre among the different lots of "Amaretti". This result is very important because it indicates that the addition of the fibre did not cause a change in the original mouthfeel of the internal paste of the cookies.

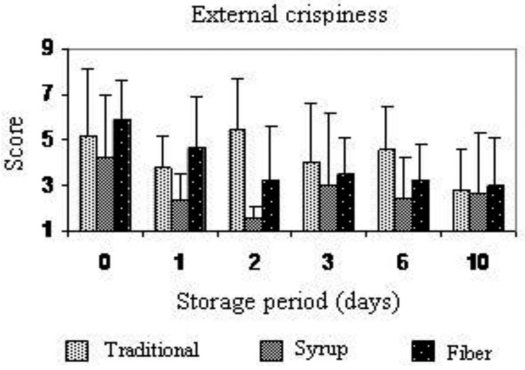


Fig. 10 - Sensorial analysis: external crispness evolution during 10 days of storage.

CONCLUSIONS

Two different ingredients were test-

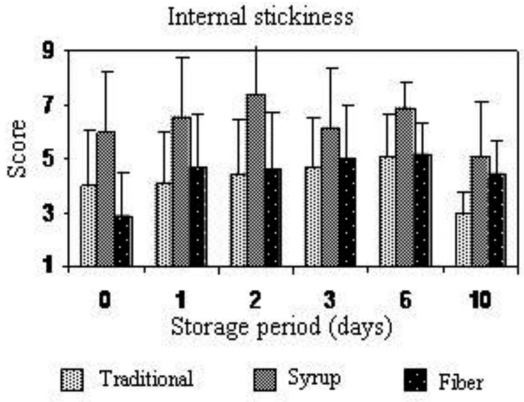


Fig. 11 - Sensorial analysis: internal stickiness evolution during 10 days of storage.

ed in the traditional recipe of "Amaretti" cookies. Glucose syrup and bamboo fibre, with known water binding properties, acted differently on the product. Although the glucose syrup obviously reduced moisture loss during the ten days of storage, it caused some modifications of the original characteristics of this regional product. The use of glucose seemed to promote water redistribution between the components of the matrix and hence sugar crystallization, which softened the crust during the first 3-4 days of storage, as pointed out by sensory analysis and the textural test.

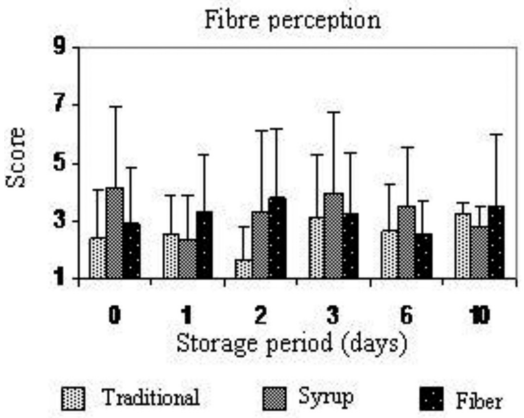


Fig. 12 - Sensorial analysis: fibre perception evolution during 10 days of storage.

The textural analysis, showed that the crust lost its crispness, probably due to water migration from the paste to the crust (due to the a_w gradient) and from the protein to the large amount of hygroscopic sugar in the amorphous form (water redistribution). In contrast, the fibre had a positive water-holding effect which limited sugar crystallisation (the discontinuity of the sorption isotherm obtained from F "Amaretti" was less pronounced than in the other two recipes). Finally, the large amount of sugar caused a more intense Maillard reaction, as shown by the colour analysis. The added fibre promoted water retention in the matrix for a longer time than the traditional recipe which helped maintain the textural differences between crust and paste. Only a small difference in colour was detected; this could be easily compensated with a small change in the time-temperature conditions during baking. The addition of these ingredients could help control moisture loss (and consequently extend the shelf-life) in high and intermediate-moisture foods. In order to get the best results, the addition of glucose and fibre should be combined with other aspects such as finding a suitable packaging solution and/or applying an edible layer between the crust and paste. Finally, experiments should be designed specifically to obtain a new optimised recipe with the best combination of factors, that would provide a partial standardization of traditional food manufacturing and extend their shelf life, while maintaining as many of the original characteristics of these typical cookies as possible.

ACKNOWLEDGEMENTS

We greatly acknowledge Pierangelo Fedeli (Chimab S.p.a., Padova) for the donation of ingredients and for the useful advice. We acknowledge Pamela Caronni who contributed to this work during her thesis project.

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Revised paper received May 26, 2007 Accepted June 14, 2007

PAPER

TOTAL PHENOLICS, MINERAL CONTENTS, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *GLYCYRRHIZA GLABRA* L. ROOTS GROWN WILD IN TURKEY

POLIFENOLI TOTALI, MINERALI, ANTIOSSIDANTI E LE PROPRIETÀ ANTIBATTERICHE DELLE RADICI DI *GLYCYRRHIZA GLABRA* L. SELVATICA CRESCIUTA IN TURCHIA

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ABSTRACT

The total phenolic content, macro and micro elements (N, P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu), antioxidant and antibacterial activities of roots of wild grown *Glycyrrhiza glabra* are reported. The amount of total phenolics was 12.88 μgGAE/mg DW in *Glycyrrhiza glabra* roots. The average N, P, K, Ca, Mg, Fe, Mn, Zn, Na and Cu composition in licorice roots was 2.80%, 175 mg/100 g,

RIASSUNTO

Sono riportati il contenuto in polifenoli totali, in macro e micro elementi (N, P, K, Ca, Mg, Na, Fe, Mn, Zn e Cu), in antiossidanti e le proprietà antibatteriche delle radici di *Glycyrrhiza glabra* selvatica. Nelle radici di *Glycyrrhiza glabra* la concentrazione di polifenoli totali era 12.88 μgGAE/mg di peso secco. La composizione media in N, P, K, Ca, Mg, Fe, Mn, Zn, Na e Cu nella liquirizia era, rispet-

- Key words: antibacterial activity, antioxidant activity, elements, *Glycyrrhiza*, phenolics -

1400 mg/100 g, 147 mg/100 g, 120 mg/100 g, 20 mg/100 g, 6 mg/100 g, 4.4 mg/100 g, 2.1 mg/100 g and 0.1 mg/100 g, respectively. The highest antioxidant activity of root extract was 88.7% at a concentration of 800 μg/mL. The methanol extracts of the roots showed antibacterial activities against *Agrobacterium tumefaciens, Bacillus cereus, Bacillus subtilus* and *Pseudomonas syringae* pv. *tomato*, but none of the water extracts of *Glycyrrhiza glabra* roots showed any antibacterial activity against the these microorganisms.

tivamente, 2,80%, 175 mg/100 g, 1400 mg/100 g, 147 mg/100 g, 120 mg/100 g, 20 mg/100 g, 6 mg/100 g, 4,4 mg/100 g, 2,1 mg/100 g e 0,1 mg/100 g. L'attività antiossidante massima dell'estratto di radice era l'88,7% ad una concentrazione di 800 μg/mL. Gli estratti metanolici presentavano attività antibatteriche contro *Agrobacterium tumefaciens, Bacillus cereus, Bacillus subtilus* e *Pseudomonas syringae* pv. *tomato*, ma nessuno degli estratti acquosi delle radici di *Glycyrrhiza glabra* presentava attività antibatterica contro questi microorganismi.

INTRODUCTION

The genus *Glycyrrhiza* includes about 20 species native to Europe, Asia, North and South America and Australia. The generic name is derived from The Greek glukos (sweet) and riza (root). The two most commonly grown species are European licorice (*Glycyrrhiza glabra* L.) and the Chinese species (*Glycyrrhiza uralensis* Fisch.); European licorice has a rich historical tradition (BOWN, 1995).

The plant is an upright, hairless perennial. It is 1.2-1.8 m tall with pinnate leaves of 9-17 leaflets and it had pale-blue, pea-like flowers (BOWN, 1995). The medicinal properties of licorice have been known for thousands of years, and the root and root extract were used in ancient China, India, Egypt, Greece and Rome (TSUKIYAMA *et al.*, 2002). The main ingredient of *Glycyrrhiza glabra* is glycyrrhizin, a substance 50-times sweeter than sucrose, with cortisone-like effects. It is a very sweet, moist, soothing herb. It is an anti-inflammatory and expectorant; it controls coughing, and has some hormonal effects. It also detoxifies and protects the liver (BAYTOP, 1996; TSUKIYAMA *et al.*, 2002).

The main *Glycyrrhiza glabra* producing countries are Turkey (the largest exporter), followed by Afghanistan, Azerbaijan, Iran, Pakistan, Russia and Syria (LANGE, 1998). The roots are boiled to extract the familiar black substance that is dried and then sold as the licorice confectionery. Licorice is also the basis for most proprietary laxatives; The extracts are also used to flavor tobacco, beer, soft drinks and pharmaceutical products. It is used as a foaming agent in beer and fire extinguishers (ATES and ERDOGRUL, 2003; BOWN, 1995).

In terms of diversity the Eastern Anatolia region is one of the richest licorice centers in Turkey. For centuries, the people in this area have been using herbal medicine, including licorice, to treat some common diseases (OZTURK *et al.*, 2000).

Recent reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases (HALLIWELL, 1997; SIES, 1993). The search for natural antioxidants is therefore very important. While earlier research on antioxidants was mostly focused on isolated pure compounds, recent studies have been directed more toward natural formulations (HAGERMAN *et al.*, 1998; HARAMAKI and PACKER, 1995; NAIK *et al.*, 2003). Plant products are also known to possess potential for food preservation and the antiox-

idant capacity of plant extracts is closely related to their phenolic compound contents (LIM and QUAH, 2007).

Recently, the growing acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to the currently available antibiotics have led researchers to investigate the antibacterial activity of some medicinal plants (KUMARASAMY *et al.*, 2002; SCRINIVASAN *et al.*, 2001). Some plants are known to produce certain bioactive molecules which react with other organisms in the environment to inhibit bacterial or fungal growth (antibiotic activity). Traditionally used medicinal plants produce a variety of compounds that are known to have therapeutic properties (BRUNETTON, 1995; CHOPRA *et al.*, 1992; COWAN, 1999).

Information is lacking on the nutritional value of *Glycyrrhiza glabra* accessions from different regions of the world. To date there have been few studies on the bioactive compounds of *Glycyrrhiza glabra*. The bioactive compounds, particularly those with antibacterial properties of wild-growing *Glycyrrhiza glabra* have not been reported in detail. The present work reports the results of an investigation on the bioactive contents, total phenolics, mineral contents and antioxidant and antibacterial activities of *Glycyrrhiza glabra* in relation its potential as a food source and natural preservative.

MATERIALS AND METHODS

Mature licorice (*Glycyrrhiza glabra* L.) plants were harvested manually from ten sites near the city of Erzurum in the Eastern Anatolia region of Turkey; the soil types at the sites were similar. At least 30 individual plants were pooled to form a single plant sample. They were put in a portable refrigerator for transportation to the laboratory (2-3 h). The samples from different locations were combined prior to analysis. Samples were dried at 50°C in an oven and the roots were then ground to a fine powder with a mortar and pestle and kept at room temperature prior to extraction for studies on the antioxidant activity and total phenolics.

The concentration of total phenolics in the ethanol extract of *Glycyrrhiza glabra* plants was determined by the Folin-Ciocalteau colorimetric method (GULCIN *et al.*, 2002). One mL of the extract solution (containing 1 mg) in ethanol was pipetted into a flask. Then, 46 mL of distilled water and 1 mL of Folin-Ciocalteau reagent were added and mixed thoroughly. The mixture was left to stand for 3 min and then 3.0 mL of 2% sodium carbonate were added. After 120 min of incubation at ambient temperature with shaking, the absorbance was measured at 760 nm. Measurements were carried out in duplicate and the calibration curve was made using gallic acid; the results are expressed as μgGAE/mg.

The antioxidant activity of *Glycyrrhiza glabra* ethanol extracts was determined according to the β-carotene bleaching method described by KAUR and KAPOOR (2002) with some modifications. Ten mg of ground sample were mixed with 10 mL of ethanol and stirred for 30 min on a magnetic stirrer. The suspension was filtered through Whatman No. 1 filter paper. Final solutions were used as stock solution for the antioxidant activity. Four mL of β-carotene solution (0.1 mg in 1 mL chloroform), 40 mg of linoleic acid and 400 mg of Tween 40 were transferred to a round-bottom flask. The mixture was then evaporated at 50 °C using a rotary evaporator to remove chloroform. One-hundred mL of oxygenated distilled water were added slowly to the residue and vigorously agitated to give a stable emulsion. Twenty-five, 50, 75, 100, 200, 400 and 800 μL of extracts were then added to 3 mL aliquots of β-carotene/linoleic acid emulsion. As the emulsion was

added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer (Thermo, Nicolet 100 UV, London, UK). The mixtures were incubated at 50°C for 90 min. The measurements were taken at 15 min intervals for 90 min. Ethanol was used as the control. A blank, devoid of β-carotene, was prepared for background subtraction. BHA was used as a standard. All samples were assayed in triplicate. The degradation rate (DR) was calculated according to first order kinetics, using the following equation based on: ln (a/b) x $1/t = DR_{sample}$ or $Dr_{standard}$ where ln is natural log, a is the initial absorbance (470 nm) at time 0, b is the absorbance (470 nm) at 100 min and t is time.

Antioxidant activity (AA) was expressed as the percentage of inhibition relative to the control, using the formula: AA = ($DR_{control}$-DR_{sample} or $_{standard}$/$DR_{control}$) x 100

Total N was determined by the micro-Kjeldahl method. In order to determine the mineral composition, samples were burned with a nitric acid and perchloric acid solution, on a hot plate, at 200°C. The absorbance of the extract was then measured using an Atomic Absorbance Spectrophotometer (Perkin Elmer, Waltham/MA, USA). The quantities of minerals were calculated with a standard curve for each element. The phosphorus content of the extract was analyzed by determining the absorbance of the yellow color, obtained from the Barton reaction, using a spectrophotometer (Thermo, Nicolet 100 UV, London, UK) at a 680 nm wavelength; the results were compared to the standard curve (JAMES, 1995).

To test the antibacterial activity, *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *tomato, Bacillus subtilis, Bacillus cereus* GC subgroup A, *Yersinia enterocolita, Vibrio cholerae* non 01, *Coryneum diphteriae, Yersinia frederiksenii, Yersinia pseudotuberculosis, Salmonella typhimurium* GC subgroup A, *Serratia liquefaciens, Pseudomonas corrugate, Xanthomonas campestris campestris, Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* were used. The bacteria, maintained on Nutrient Agar (Merck, Darmstadt, Germany) were supplied by The Microbiology Laboratory of The Agricultural Faculty of Ataturk University, Erzurum, Turkey. The identity of the bacteria used in this study was confirmed by The Microbial Identification System in the Biotechnology Application and Research Center at Ataturk University. The dried and powdered licorice root materials (400 g) were extracted successively in a Soxhlet extractor with methanol (MeOH) for 72 h at a temperature not exceeding the boiling point of the solvent (OZTURK and ERCISLI, 2007). The extracts were filtered using Whatman filter paper (No. 1) and then concentrated in vacuo at 40°C using a rotary evaporator. The residues obtained were used for the antibacterial tests. Antibacterial activities were determined using the disk diffusion method in which 100 μL of suspension containing 10^8 CFU/mL of bacteria were spread on nutrient agar (NA) medium. Sterile, 6 mm diameter filter paper discs were impregnated with 10 μg of sterile test material and placed on nutrient agar. Negative controls were prepared using the same solvents as those used to dissolve the plant extracts. Ofloxacin (5 μg/disc), sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disc) and/or netilmicin (30 μg/disc) were used as positive standards. The bacteria-inoculated plates were incubated at 27°C for 24 h. The diameter of the clear zone around the disc was measured and the antibacterial activity is expressed in mm. Five discs per plate and three plates were used, and each test was run in triplicate (DJIPA *et al.*, 2000).

The minimum inhibition concentration (MIC) values were also determined. The inocula of bacteria were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. *Glycyrrhiza glabra*

root extracts dissolved in 0.5% dimethylsulfoxide (DMSO) were first diluted at the highest concentration (500 μg/mL) to be tested, and then serial two-fold dilutions were made in a concentration range from 3.90 to 500 μg/mL in 10 mL sterile test tubes containing nutrient broth. The MIC values of extracts against bacterial strains were determined based on a micro-well dilution method (OZTURK and ERCISLI, 2007). The 96-well plates were prepared by dispensing 95 μL of nutrient broth and 5 μL of the inoculum into each well. One-hundred μL of the extracts initially prepared at the 500 μg /mL concentration were added to the first wells. One-hundred μL of the serial dilutions were then transferred into six consecutive wells. The last well containing 195 μL of nutrient broth without any compounds and 5 μL of the inoculum on each strip was used as the negative control. The final volume in each well was 200 μL. Maxipime (Bristol-Myers Squibb, New York, USA) at the concentration range of 500-3.9 μg /mL was prepared in nutrient broth and used as the standard drug for positive control. The contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at the appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., Highland Park, VT, USA) and confirmed by plating 5 μL samples from clear wells onto nutrient agar medium. The extract tested in this study was screened twice against each organism. The MIC of each extract was taken as the lowest concentration that showed no growth (OZTURK and ERCISLI, 2007).

All reagents were of analytical grade and obtained from Sigma Chemical Co. (St Louis, MO, USA).

The experiment was a completely randomized design with four replications. Data were subjected to analysis of variance (ANOVA) and the means were separated by a Duncan multiple range test at the $p<0.05$ significant level.

RESULTS AND DISCUSSION

The antioxidant activity of *Glycyrrhiza glabra* roots is reported in Table 1. The differences among *Glycyrrhiza glabra* root extract concentrations and BHA concentrations were statistically significant ($p<0.05$). Antioxidant activity was found to be 28.3% at 25 μg/mL extract concentrations and increased to 66.4%

Table 1 - Antioxidant activity in *Glycyrrhiza glabra* root extract and BHA.

BHA Concentration (μg/mL)	Antioxidant activity (%)	Root extracts Concentration (μg/mL)	Antioxidant activity (%)
25	31.7±3.7^{d}	25	28.3±5.5^{c}
50	63.3±5.7^{c}	50	39.6±6.6^{c}
75	80.4±6.9^{b}	75	58.5±7.3^{b}
100	98.4±8.7^{a}	100	66.4±7.7^{b}
		200	83.1±8.3^{a}
		400	87.4±8.7^{a}
		800	88.7±8.4^{a}

Values (on dry basis) represent, the means of a triplicate determination (n=3) ±SD. Values in a column with different superscripts are significantly different at $p<0.05$.

at 100 μg/mL and finally reached 88.7% at 800 μg/mL in *Glycyrrhiza glabra* root extract. The antioxidant activity of BHA was 31.7% at 25 μg/mL and reached nearly 100% (98.4%) at the 100 μg/mL concentration. This suggests that *Glycyrrhiza glabra* root extract may contain high concentrations of active compounds. OKADA *et al.* (1989) reported that *Glycyrrhiza glabra* roots from Russia and China had antioxidant constituents. The present results show that *Glycyrrhiza glabra* roots have a strong antioxidant capacity and may be important for both the human diet and food safety when mixed with the other foods. The antioxidant activity of *Glycyrrhiza glabra* roots was previously reported in Ayurvedic medicine in India (NAIK *et al.*, 2003). HARAGUCHI *et al.*, (1998a) reported that the other *Glycyrrhiza* species, *Glycyrrhiza inflata* also had antioxidative and superoxide scavenging activities. Studies on the antioxidant content of *Glycyrrhiza* plants are being carried out in various parts of the world. As expected, there is an enormous variation in the antioxidant content due to difference in climatic conditions and the plants used. From these results, it can be suggested that the consumption of *Glycyrrhiza glabra* roots may offer some dietary benefits since they contain antioxidant constituents, which can protect against lipid peroxidation and scavenge free radicals. This report further suggests that *Glycyrrhiza glabra* may have beneficial chemopreventive effects in addition to being a potential new source of natural antioxidants.

The total phenolic compounds were determined by the Folin-Ciocalteau method. This method provides a simple, inexpensive, quick and useful estimate of total phenolics. Phytochemicals, especially phenolics, are thought to be most important bioactive compounds for health benefits. Plant extracts, which contain different classes of polyphenols are very attractive not only in modern phytotherapy but also in the food industry. Therefore, the total phenolic content of *Glycyrrhiza glabra* root extracts was investigated.

Total phenolics in *Glycyrrhiza glabra* roots were found to be 12.88 μgGAE/mg DW. The results for total phenolics of *Glycyrrhiza glabra* root extracts clearly show that it is one of the richest phenolic sources. HATANO *et al.* (2000) previously reported that *Glycyrrhiza glabra* roots are rich in phenolic constituents and the variability in phenolic content in different plants could be due to the plants used, environmental factors and the collection period. Since phenolics are one of the most important groups of compounds acting as primary antioxidants, it was reasonable to determine the total phenolic content in *Glycyrrhiza glabra*. Phenolics have also played an important role against chronic diseases (NIJVELDT *et al.*, 2001).

The mineral content of *Glycyrrhiza glabra* root is made up of the elements N, P, K, Ca, Mg, Fe, Mn, Zn, Na and Cu in varying concentrations which are reported in Table 2.

To date there have been no reports in the literature related to the mineral composition of *Glycyrrhiza glabra* root; this study is the first in this area. The mineral composition of the plants depends not only on the species or variety,

Table 2 - Mineral contents (mg/100g) in *Glycyrrhiza glabra* roots.

N (%)	P	K	Ca	Mg	Fe	Mn	Zn	Na	Cu
2.80±0.4	175±23	1400±169	147±16	120±14	20±3	6±1	4.4±1	2.1±0.3	0.1±0.0
Values (on dry basis) represent the means of a triplicate determination (n=3) ±SD.									

Table 3 - Antibacterial activity of *Glycyrrhiza glabra* extracts against some bacteria.

Bacterial species	Inhibition zone in diameter (mm) of *Glycyrrhiza glabra* extracts (10 µg/disc)	Water extract	Negative control MEOH	Positive control (mm)			MIC (µg/mL)	
				Net	Sfc	Ofx	extract	Standard drug Maxipime
Pseudomonas syringae pv. *syringae*	-	-	-	16	-	16	3.90	250
Pseudomonas syringae pv. *tomato*	8	-	-	20	-	-	125	250
Bacillus subtilis	7	-	-	30	40	26	3.90	7.81
Bacillus cereus	8	-	-	19	19	21	62.50	31.25
Yersinia enterocolita	-	-	-	-	19	-	15.62	31.25
Vibrio cholerae	-	-	-	16	39	21	125	62.50
Coryneum diphteriae	-	-	-	-	-	-	3.90	-
Yersinia frederiksenii	-	-	-	12	19	18	7.81	31.25
Yersinia pseudotuberculosis	-	-	-	17	12	16	125	31.25
Salmonella typhimurium	-	-	-	-	9	-	31.25	-
Serratia liquefaciens	-	-	-	14	-	10	15.62	62.50
Pseudomonas corrugata	-	-	-	13	10	23	3.90	-
Xanthomonas compestris compestris	-	-	-	9	15	15	125	500
Agrobaceterium tumefaciens	7	-	-	16	25	23	3.90	-
Pseudomonas aeruginosa	-	-	-	14	12	17	125	250

but also on the growing conditions such as soil type and geographical location.

The antibacterial activity of *Glycyrrhiza glabra* root against several bacteria is shown in Table 3. The *Glycyrrhiza glabra* root extract showed antibacterial activity against *Pseudomonas syringae* pv. *tomato*, *Bacillus subtilus*, *Bacillus cereus* and *Agrobaceterium tumefaciens* (7-11 mm inhibition zone). However, none of the aqueous extracts showed any antibacterial activity against the microorganisms studied. The extracts did not show any antibacterial activity against *Coryneum diphteriae*, *Pseudomonas aeruginosa*, *Pseudomonas corrugate*, *Pseudomonas syringae pv. syringae*, *Salmonella typhimurium*, *Serratia liquefaciens*, *Vibrio chlororae*, *Yersinia enterocolita*, *Yersinia frederiksenii*, *Yersinia pseudotuberculosis* or *Xanthomonas compestris compestris*.

MIC values for the methanol extract of *Glycyrrhiza glabra* and standard drug (Maxipime) were in the range of 3.90-125 µg/mL and 7.81-500 µg/mL, respectively (Table 3). *Pseudomonas syringae* pv. *syringae*, *Bacillus subtilis*, *Coryneum diphteriae*, *Pseudomonas corrugate* and *Agrobaceterium tumefaciens* were the most sensitive to the plant extract with MIC values lower (3.90 µg/mL) than the standard drug. *Pseudomonas syringae* pv. *tomato*, *Yersinia enterocolita*, *Yersinia frederiksenii*, *Serratia liquefaciens*, *Pseudomonas aeruginos-*

sa and *Xanthomonas compestris compestris* were also sensitive with MIC values that were lower than the standard antibiotic (Maxipime).

For centuries, the root and rhizome of *Glycyrrhiza spp.* have been used as a medicine because of its wide-ranging therapeutic properties. TSUKIYAMI *et al.* (2002) reported that licochalcone obtained from *Glycyrrhiza spp.* roots possessed antibacterial activity particularly against all gram-positive bacteria, including *Bacillus* spp. It was previously reported that *Glycyrrhiza glabra* root extracts showed various antibacterial activities against certain microorganisms tested (ATES and OZDOGRUL, 2003; HARAGUCHI *et al.*, 1998b; OKADA *et al.*, 1989). The genera *Bacillus* are a well known spore-forming bacteria and *Bacillus cereus* is a toxin-producing bacteria. Therefore the root of *Glycyrrhiza glabra* could be used to prevent food contamination since the recent trend in food processing is to avoid the use of chemical preservatives. Now, natural antimicrobial alternatives are needed.

In conclusion, wild *Glycyrrhiza glabra* plants have a high total phenolic content and show high antioxidant and antibacterial activities. Wild *Glycyrrhiza glabra* plants could provide a new source of antioxidant and antibacterial agents and can be used for food and medicinal purposes. Planting date and variety factors must also be studied in an attempt to improve and domesticate *Glycyrrhiza glabra* as a major agricultural crop.

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Revised paper received April 23, 2007 Accepted July 3, 2007

SHORT COMMUNICATION

PARTIAL CHARACTERIZATION OF STARCH IN FLOURS OF ANCIENT WHEAT AND WILD PROGENITOR ACCESSIONS

CARATTERIZZAZIONE PARZIALE DELL'AMIDO IN FARINE DI GRANO ANTICO E DI LORO PROGENITORI SELVATICI

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ABSTRACT

The starch properties of 17 ancient wheats and wild progenitors (*Triticum monococcum* L. var. *monococcum*, *T. dicoccum* Sch., *Aegilops speltoides* Tausch. var. *ligustica*, *A. speltoides* Tausch var. *speltoides*, *T. monococcum* L. var. *boeoticum*, and *T. monococcum* L. var. *urartu*) collected in Turkey were evaluated. The study demonstrated that some of the starch properties (birefringence, X-ray diffraction patterns) were comparable to those of durum wheat. There was large vari-

RIASSUNTO

Sono state studiate le proprietà dell'amido di 17 tipologie di grano antico e dei progenitori selvatici raccolte in Turchia (*Triticum monococcum* L. var. *monococcum*, *T. dicoccum* Sch., *Aegilops speltoides* Tausch. var. *ligustica*, *A. speltoides* Tausch var. *speltoides*, *T. monococcum* L. var. *boeoticum*, e *T. monococcum* L. var. *urartu*). Lo studio ha dimostrato che alcune proprietà dell'amido (birifrangenza, andamenti della diffrazione ai raggi X) erano comparabili a quelle del grano duro. Fra i campioni di

- Key words: ancient wheats, birefringence, DSC, RVA, starch -

ation in the Rapid Viscoanalyzer viscosity values among the ancient wheat samples. The gelatinization temperatures (onset and midpoint) of the ancient wheat samples were slightly higher than those of the durum wheat sample as determined by differential scanning calorimetry. The results might be useful for breeders and industry.

grano antico si sono osservate ampie variazioni dei valori di viscosità, analizzate mediante Viscosimetro Rapido, fra i campioni di grano antico. Le temperature di gelatinizzazione (all'inizio e a metà del processo) dei campioni di grano antico erano leggermente superiori a quelle del grano duro, come determinato con la calorimetria differenziale a scansione. I risultati ottenuti potrebbero essere utili per i genetisti e l'industria.

INTRODUCTION

In the last two decades, there has been considerable interest in the consumption of ancient wheats, especially targeted for organic and health food markets. Some of the interest arises from claims that ancient wheats may be low in allergenicity (ABDEL-AAL *et al.*, 1998a) and high in certain nutrients (ABDEL-AAL *et al.*, 1995; RANHOTRA *et al.*, 1996; PIERGIOVANNI *et al.*, 1997). Eincorn, emmer and spelt are among the earliest cultivated wheats and are commonly referred to as "ancient" wheats. They are hulled and commonly used as animal feed. Glumes should be removed prior to use in human food products (ABDEL-AAL *et al.*, 1998b).

It is assumed that there are three main genomes involved in wheat evolution; namely the A, B, and D genomes. Many scientists agree that the progenitors of these genomes are *Triticum monococcum* (var. *boeoticum* and var. *urartu*), *Aegilops speltoides* (var. *speltoides* and var. *ligustica*) and *Aegilops tauschii* Coss., respectively. Apart from these three major progenitors, there are several other wild relatives. Cultivated wheats fall into one of three ploidy levels. The first group is diploid (AA) cultivated einkorn (*T. monococcum* var. *monococcum*) with 14 chromosomes which is closely related to the wild progenitor *T. monococcum* (wild einkorn). This is a semi-wild group with a very small area of cultivation in Turkey; it is almost extinct in other parts of the world. The second group is the tetraploid (AABB) wheats with 28 chromosomes that are called, in a general sense, "the hard wheats". They are presumed to have evolved from the preliminary wild relative *Triticum dicoccoides* Koern. (wild emmer) as a result of natural hybridization between *T. monococcum* (AA) and *A. speltoides* (BB). The tetraploid group also contains a semi-wild cultivated wheat species *T. dicoccum* (cultivated emmer) which has a small area of cultivation in the world. The third group is hexaploid (AABBDD) with 42 chromosomes. It is the most widely cultivated group of wheats in the world, known as "the bread wheats". This group is assumed to be a product of hybridization between *T. dicoccoides* (AABB) and *A. tauschii* (DD) (KIMBER and SEARS, 1987). Although the evolutionary process is rather complicated and not as simple as indicated above, it is important to know the major actors playing in the evolution. There are several records concerning emmer and einkorn cultivation in Turkey. Reports indicate that emmer occupied almost 2% of the total cultivated area of Anatolia at the end of the first quarter of the 20th century (ZHUKOVSKY, 1951). The total acreage of cultivated einkorn and emmer at that time was estimated to have been 137,300 ha (KARAGOZ, 1996), while it is now about 6,000 ha (ANONY-

MOUS, 2003) in Turkey. Spelt wheat is not cultivated in Turkey.

Turkey is located at a significant position in the world from the view point of plant genetic resources and genetic diversity (KARAGOZ, 1998). It is agreed that two of the centers of diversity and centers of origin, the Near Eastern and the Mediterranean, overlap in Turkey. It is accepted by many scientists that wheat and barley were first domesticated in the Fertile Crescent of the Near East. The Fertile Crescent is defined as a semi-circle covering Palestine, Israel, the western part of Syria, southeastern Turkey, eastern Iraq and the western part of Iran. In addition to the previous information, several papers were recently published claiming that wheat was first domesticated in Karacadag in Turkey. The scientists came to this conclusion after analyzing thousands of accessions collected from different regions of the world including Karacadag (HEUN *et al.*, 1997; DIAMOND, 1997; NESBIT and SAMUEL, 1998; LEV-YADUN *et al.*, 2000).

There are some publications on grain characteristics, processing requirements, and nutritional and health attributes of ancient wheats and their potential applications in wheat-based food products (ABDEL-AAL *et al.*, 1998b). In general, spelt may contain higher levels of protein, soluble dietary fiber and minerals depending on the genotype. Several reports have indicated that spelt wheat has good potential in a variety of cereal products including bread, pasta and breakfast cereals (RANHOTRA *et al.*, 1996; PIERGIOVANNI *et al.*, 1997; ABDEL-AAL *et al.*, 1997; MARCONI *et al.*, 1999; BONAFACCIA *et al.*, 2000). Einkorn is reported to have high levels of protein, carotenoids and phosphorus (ABDEL-AAL *et al.*, 1995; BORGHI *et al.*, 1996; CORBELLINI *et al.*, 1999; D'EGIDIO *et al.*, 1993).

Although, there are some research studies on the chemical composition and breadmaking quality of ancient wheats, to the best of our knowledge, there are only a few publications on the properties of their starch (LØJE *et al.*, 2003). In this study, the properties of ancient wheat starch (e.g. birefringence, X-ray diffraction, differential scanning calorimetry and pasting properties) were investigated in order to evaluate the potential effects of these starches on food properties.

MATERIALS AND METHODS

Materials

The ancient wheat samples and wild progenitors used in this study were obtained from the Germplasm Collection of the Central Research Institute for Field Crops (Ankara, Turkey) which were collected from different sites in Turkey and are listed in Table 1. The *A. speltoides* samples were growing naturally and the rest of the samples were taken from local farmers. The 1000 kernel weight was determined by counting the number of seeds in 20 g of grain. The samples were prepared by manual dehulling, grinding in a coffee grinder and sifting through a 106 μm sieve to remove branny particles. Ground sample of Canadian western amber durum wheat (CWAD-2) was used as a control.

Birefringence studies

Birefringence was evaluated in water-glycerol (50:50) suspensions (v/v) of ground samples according to KOKSEL *et al.* (1993). Bright-field and polarized-light microscopic examinations were performed using a Zeiss microscope (Kameramikroskop Ultraphot III, Bonn, Germany) and photographed on 35 mm Ilford FP4 film (ASA 125).

X-ray diffractometry

The X-ray diffraction patterns of ground samples were obtained on a

Philips X-ray diffractometer, PW 1140 (Scientific Equipment Dep., N.V. Philips, Gloeilampen-fabrieken, The Netherlands) equipped with a monochromator that selects the Kα radiation from a copper target generated under 40 kV and 18 mA. Diffraction patterns were recorded at a diffraction angle (2θ) of 5-35° at a scan speed of 2°/min (KOKSEL *et al.*, 1993).

Differential scanning calorimetry (DSC)

The ground samples were weighed (2.3-3.3 mg) into DSC pans and wetted by adding distilled water. The ratio of dry sample to water was around 1:3. The DSC pans were sealed and allowed to equilibrate overnight in a refrigerator prior to analysis. The samples were placed in the standard DSC cell with an empty cell used as a reference. The DSC cell was heated at a rate of 10°C/min to 100°C using a Dupont Model 2200 Thermal Analyzer (Dupont TA Instruments, Newcastle, DE, USA) with a standard DSC cell. Enthalpies, onset temperatures and peak temperatures were computed automatically via the standard analysis software which was supplied with the instrument.

Table 1 - Species identification and source of the wheat materials.

Accession Number	Species	Location	Altitude (m)	1000 kernel weight	
				with glume	without glume
TUR 01336	*Aegilops speltoides* var. *ligustica*	Sanliurfa, Hilvan	550	11.30	8.42
TUR 00313	*Aegilops speltoides* var. *speltoides*	Adiyaman, Bardakci Vilage	585	12.58	12.22
	Average *(A. speltoides)*			11.94	10.32
TUR 00342	*Triticum monococcum* var. *boeoticum*	14 km south of Adiyaman	515	36.90	14.68
TUR 00630	*Triticum monococcum* var. *boeoticum*	Adiyaman, Besele Vilage	670	35.17	14.43
TUR 02571	*Triticum monococcum* var. *boeoticum*	Gaziantep, Oguzeli	635	37.32	15.24
TUR 02335	*Triticum monococcum* var. *monococcum*	Cankiri, Cerkes.	1200	36.19	24.95
TUR 02574	*Triticum monococcum* var. *urartu*	Gaziantep, Oguzeli	700	36.92	15.97
TUR 01408	*Triticum monococcum* var. *urartu*	Sanliurfa, Ovacik	525	37.76	17.61
	Average *(T. monococcum)*			36.71	17.15
TUR 02416	*Triticum dicoccum*	Sinop, Ormantepe Vilage	950	37.36	34.94
TUR 02418	*Triticum dicoccum*	Sinop, Ormantepe Vilage	950	33.28	31.24
TUR 02427	*Triticum dicoccum*	Sinop, Hacimahmutlu Vilage	950	37.73	33.77
TUR 02432	*Triticum dicoccum*	Sinop, Ortakoy Vilage	1000	35.48	32.64
TUR 02439	*Triticum dicoccum*	Sinop, Sarikadi Vilage	1100	38.62	33.99
TUR 02453	*Triticum dicoccum*	Sinop, Golyeri Vilage	1200	33.46	31.46
TUR 02456	*Triticum dicoccum*	Sinop, Beyardic Vilage	1100	34.45	32.38
TUR 02504	*Triticum dicoccum*	Sinop, Beyardic Vilage	1100	32.80	30.83
TUR 02540	*Triticum dicoccum*	Corum, Campinar Vilage	1600	37.87	35.59
	Average *(T. dicoccum)*			35.67	32.98
	Overall Average			33.25	24.73

Pasting properties

Starch pasting properties of ancient wheat flours were determined using a Rapid ViscoAnalyzer (RVA 4, Newport Scientific, Warriewood, Australia). In this assay, 3 g (14% moisture basis) of sample were dispersed in 25 mL distilled water in an aluminum sample canister. The RVA pasting curve was obtained by using an 11 min test profile: initial equilibrium at 50°C for 1 min 20 sec, heating to 95°C over 3 min 25 sec, holding at 95°C for 2 min 30 sec, cooling to 50°C over 3 min 45 sec. Cold viscosity, peak viscosity, through, breakdown and final viscosity values were evaluated with the data analysis software (Thermocline for Windows, Newport Scientific, Warriewood, Australia).

RESULTS AND DISCUSSION

Some of the bright-field and polarized-light micrographs of selected ancient wheat and wild progenitor samples are presented in Fig. 1 (A-F). There were large lenticular and small spherical granules in the micrographs of ancient wheat samples. Bright-field and polarized-light microscopy studies showed that the appearance of starch in ancient wheat samples was similar to that of the durum wheat which was used as control. As expected, all of the starch granules of the ancient wheat samples displayed a clear Maltese cross under polarized light, since they were not heat treated.

The X-ray diffractograms of selected ancient wheat samples and the control are presented in Fig. 2. The A-type X-ray diffraction patterns of the ancient wheat samples were similar to that of the durum wheat as well as the ones reported in the literature for a common wheat (KOKSEL *et al.*, 1993; HOSENEY, 1994; ZOBEL, 1988a,b). Their spectra showed three distinct, but broad, peaks between 2θ angles of 14 and 16°, 16 and 19°, and 21.5 and 25° (Fig. 2). When the X-ray spectra of these ancient wheat samples were evaluated, it was observed that an additional (minor) peak appeared at about 2θ = 20° with d spacing of 4.4 Å in the X-ray spectra of some of the ancient wheat samples (e.g., Fig. 2 A, C, E). It has been reported that naturally occurring fatty acids and phospholipids can complex with the amylose fraction and result in V structures due to heat treatment. V-type X-ray diffraction patterns had three peaks at 12, 6.8, and 4.4 Å. The peak at 4.4 Å often appears as a first indication of V-complex formation (HOSENEY, 1994). Although the ancient wheat samples used in this study were not heat-treated, they had this minor peak around 2θ = 20°. Further work is necessary to clarify this matter.

The principle of DSC measurement is to compare the rate of heat flow to the sample and to a reference material, both of which are subjected to identical temperature changes. The phase changes in the sample which are associated with absorption or evolution of heat causes a change in the differential heat flow which is then recorded as a peak. The area under the peak is related to the energy involved in the phase change. The DSC results of the ancient wheat samples and control are presented in Table 2. The results indicated that ancient wheat samples had onset temperatures in the range of 56.4°-63.0°C and midpoint temperatures in the range of 63.8°-68.7°C. The highest onset and midpoint temperatures were obtained for the *T. dicoccum* sample (TUR 02453) and the lowest onset and midpoint temperatures were obtained for *Aegilops speltoides* var. *speltoides* (TUR 00313). The gelatinization temperatures (onset and midpoint) of the ancient wheat samples were slightly higher than for the common wheat sample as determined by DSC (GHIASI *et al.*, 1982; NODA *et*

al., 2001). LØJE *et al.*, (2003) also reported that the gelatinization temperature of the einkorn and emmer samples were generally higher than the common wheat sample as determined by amylograph. The enthalpy values of durum wheat and ancient wheat samples were in the range of 3.6-7.5 J/g (Table 2). These enthalpy values are lower than those reported in the literature for common wheat (NODA *et al.*, 2001; ERDOGDU *et al.*, 1995). However, the values reported in the literature were obtained by using isolated starch samples, while in the present study ground meal was used due to the limited quan-

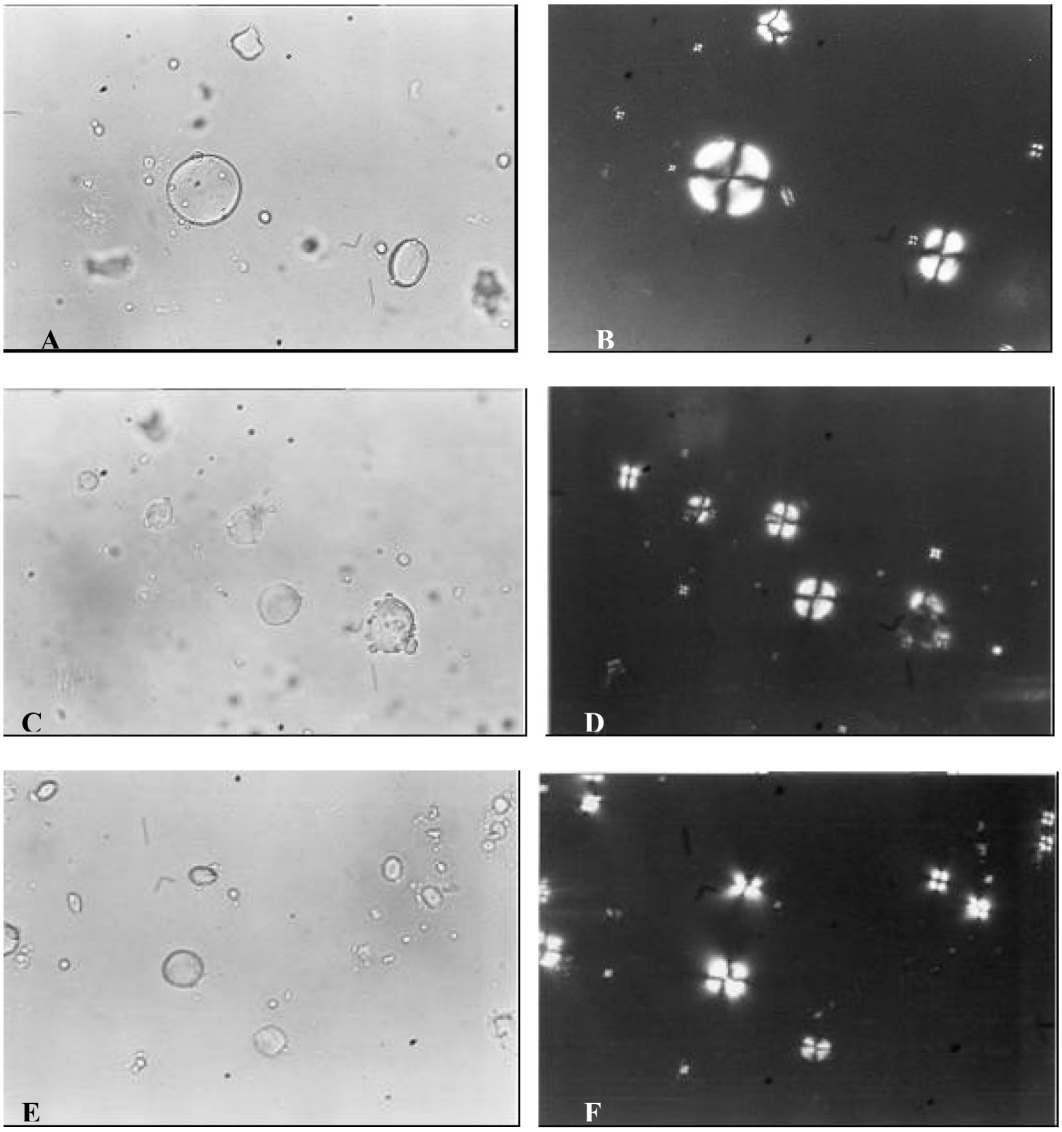


Fig. 1 - Bright-field (left) and polarized-light (right) micrographs of ancient wheat samples: TUR 01336 (A and B), TUR 01408 (C and D), TUR 02504 (E and F).

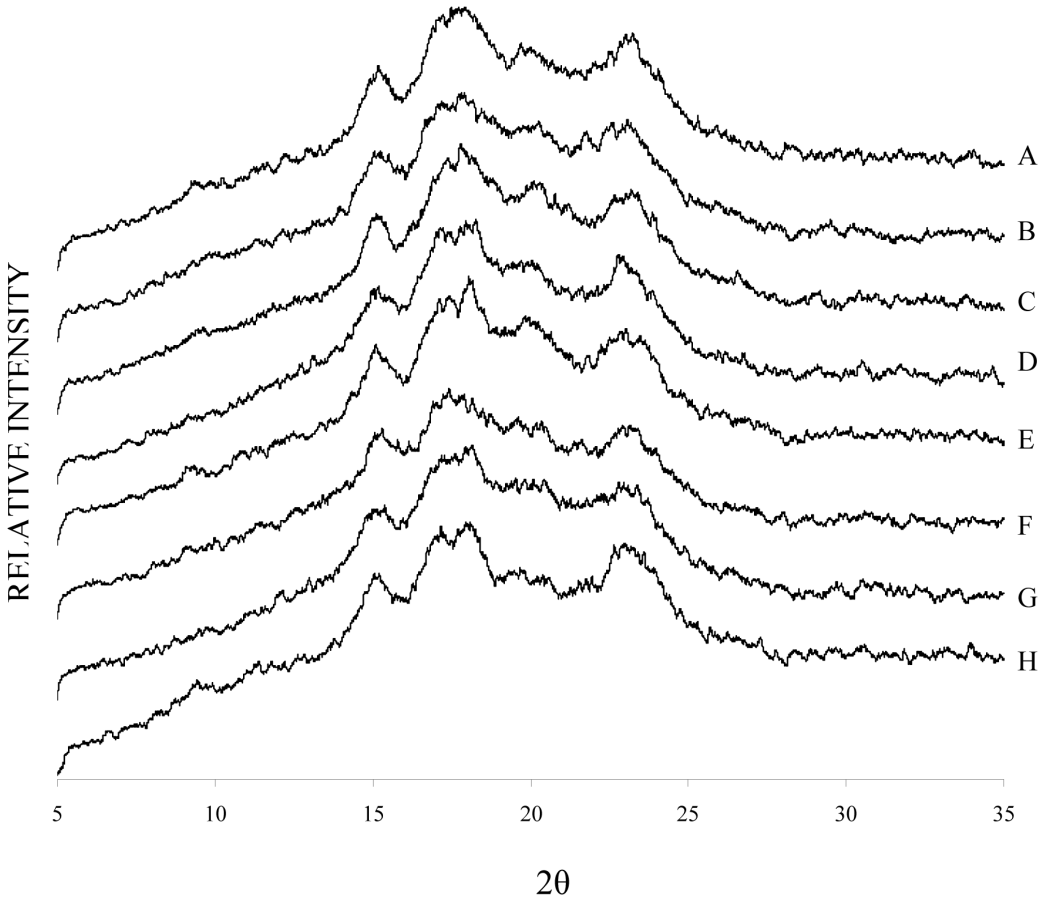


Fig. 2 - X-ray diffractograms of ground ancient wheat samples: A: TUR 01336, B: TUR0313, C: TUR 00342, D: TUR 02335, E: TUR 01408, F: TUR 02416, G: 02504, H: Durum wheat (Control).

Table 2 - Differential scanning calorimetry characteristics of some ancient wheat samples.

Sample	T_o, Onset Temp (°C)	T_p, Midpoint (°C)	ΔH, Enthalpy (J/g)
A. speltoides var. *ligustica*	60.9	66.5	4.0
A. speltoides var. *speltoides*	56.4	63.8	6.4
Average *(A. speltoides)*	58.7	65.2	5.2
T. monococcum var. *boeoticum*	61.0	67.5	4.4
T. monococcum var. *monococcum*	59.9	65.5	6.0
T. monococcum var. *urartu*	61.6	65.0	7.5
Average *(T. monococcum)*	60.8	66.0	6.0
T. dicoccum	63.0	68.7	3.7
T. dicoccum	59.7	65.0	4.4
Average *(T. dicoccum)*	61.4	66.9	4.1
Overall Average	60.4±2.06	66.0±1.68	5.2±1.43
T. durum (Control)	54.2	61.6	4.8

tity of each sample available. The relative amount of starch in ground ancient wheat samples is lower than that in isolated starch samples, which is probably the main reason for the lower enthalpy values of the former.

RVA pasting properties of ancient wheat samples are summarized in Table 3. There was a large variation among the viscosity values of the different ancient wheat samples. The highest peak, trough and final viscosity values were obtained for the *T. monococcum* var. *urartu* sample (TUR 01408) and the lowest peak, trough and final viscosity values were obtained for the *A. speltoides* var. *ligustica* sample (TUR 01336). Generally, *T. monococcum* and *T. dicoccum* samples had similar peak, trough and final viscosity values which were higher than those of *A. speltoides* samples. The samples used in the present study varied in their 1000 kernel weights (Table 1). Some of them had very thin, small kernels (e.g., *A. speltoides* samples), while others had larger, plump kernels. It has been reported that large, dense wheat kernels normally have a higher ratio of endosperm to nonendosperm components than do smaller, less dense kernels (HALVERSON and ZELENY, 1988). Therefore, for samples with smaller kernels, the level of bran in the whole-meals will be higher than that in the larger kernels. Because of the higher ratio of endosperm, larger kernels in the present study have a higher level of starch content, leading to higher RVA viscosity values. Some of the ancient wheat samples had RVA pasting profiles similar to that of the durum wheat (control) while some of them had quite low RVA viscosity values, probably due to their physical properties (thin, small kernels)

Table 3 - Starch pasting properties of ancient wheat samples.

Species	Peak Viscosity (RVU)	Trough Viscosity (RVU)	Breakdown Viscosity (RVU)	Final Viscosity (RVU)	Setback Viscosity (RVU)
A. speltoides var. *ligustica*	38	29	9	69	40
A. speltoides var. *speltoides*	58	42	17	84	42
Average *(A. speltoides)*	48.0	35.5	13.0	76.5	41.0
T. monococcum var. *boeoticum*	70	60	10	126	66
T. monococcum var. *boeoticum*	61	51	10	107	56
T. monococcum var. *boeoticum*	72	56	16	125	70
T. monococcum var. *monococcum*	81	68	13	139	72
T. monococcum var. *urartu*	66	50	16	114	65
T. monococcum var. *urartu*	100	72	29	155	84
Average *(T. monococcum)*	75.0	59.5	15.7	127.7	68.8
T. dicoccum	68	58	10	116	57
T. dicoccum	75	64	11	123	59
T. dicoccum	80	67	13	125	58
T. dicoccum	81	60	21	121	61
T. dicoccum	78	64	14	125	61
T. dicoccum	69	58	11	112	54
T. dicoccum	75	61	14	124	63
T. dicoccum	57	49	9	96	47
T. dicoccum	84	54	30	113	59
Average *(T. dicoccum)*	74.1	59.4	14.8	117.2	57.7
Overall Average	71.4	56.6	14.9	116.1	59.6
T. durum (Control)	143	88	55	189	100

and chemical composition (low starch content) of their kernels. Further work is necessary to elucidate the effects of physical properties and chemical composition of kernels on pasting properties. Some of the ancient wheat samples (Table 3; TUR 00342, TUR 02335, TUR 02416, TUR 02418, TUR 02427, TUR 02439, TUR 02453) had favorable hot paste stability (low RVA breakdown values) compared to that of the durum wheat sample, which might be desirable in certain food products.

CONCLUSIONS

Turkey is of remarkable importance in terms of the genetic resources of many economically significant plant species, especially ancient wheats. Although there are some publications on grain characteristics, processing requirements and nutritional and health attributes of ancient wheats, the information on various characteristics of ancient wheats is still incomplete. In the present study, the starch properties of ancient wheat samples and wild progenitors collected from different sites in Turkey were evaluated. Starch is the major component of cereals and affects the properties (texture, mouth-feel, etc.) of cereal-based foods to a large extent. The food industry requires starches with diverse functional properties. This study demonstrated that some of the properties (birefringence, X-ray diffractograms, DSC enthalpy values) of starch in most of the ancient wheat samples were comparable with those of the durum wheat studied. However, the ancient and durum wheat samples studied were quite different in some of their other properties (RVA pasting properties). Therefore, future research should examine minor differences in starch structure and functionality of ancient wheats which might hold exciting possibilities for the food industry.

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Revised paper received July 1, 2007 Accepted August 6, 2007

SHORT COMMUNICATION

REDUCTION OF OIL UPTAKE IN DEEP FAT FRIED CHICKEN NUGGETS USING EDIBLE COATINGS BASED ON CASSAVA STARCH AND METHYLCELLULOSE

RIDUZIONE DELL'ASSORBIMENTO DI GRASSI IN MEDAGLIONI DI POLLO FRITTI MEDIANTE IMMERSIONE IN OLIO CON L'IMPIEGO DI RIVESTIMENTI COMMESTIBILI COSTITUITI DA AMIDO DI CASSAVA E METIL CELLULOSA

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ABSTRACT

The aim of this study was to evaluate the effect of edible coatings based on methylcellulose (MC) and cassava starch (CS) to reduce oil uptake and improve water retention of chicken nuggets during deep fat frying. Edible coatings were prepared with 1 g of MC/100 g solution and 4 g of CS/100 g solution, with 25 or 55 g glycerol/100 g biopolymer. These solutions were applied to nugget samples before battering. Pre-fried and fried nuggets were analyzed to determine lipid and water

RIASSUNTO

Lo scopo di questo studio era quello di valutare l'effetto di un rivestimento costituito da metilcellulosa (MC) e amido di cassava (CS) sulla riduzione dell'assorbimento di olio e sul miglioramento della ritenzione di acqua in medaglioni di pollo durante la frittura per immersione. I rivestimenti commestibili erano preparati con una soluzione contenente 1 g di MC/100 g e 4 g di CS/100 g e con un biopolimero contenente 25 o 55 g di glicerolo/100 g. Queste soluzioni venivano applicate ai medaglioni pri-

- Key words: cassava starch, chicken nuggets, deep-fat frying, edible coating, methylcellulose -

contents. Color and texture were also measured in the fried nuggets. In general, there was no effect of the two concentrations of plasticizer of either of the biopolymers on the water retention of whole nuggets. But, higher oil uptake reduction, and consequently, lower lipid content was observed on nuggets coated with CS and 25% plasticizer. The coated samples were darker and had a brighter yellow color when compared with the control. There was also a significant decrease in the shearing force of the fried coated samples, indicating reduced hardness of these samples.

ma della pastella. I medaglioni venivano analizzati per determinarne il contenuto in lipidi ed acqua, prima e dopo la frittura. Sono stati inoltre valutati il colore e la consistenza dei medaglioni fritti. In generale, non si osservava alcun effetto delle due diverse concentrazioni di plasticizzante dei biopolimeri sulla ritenzione di acqua da parte dei medaglioni. D'altra parte, una riduzione dell'assorbimento di olio e, di conseguenza, un minore contenuto in lipidi sono stati osservati sui medaglioni con CS e con il 25% di plasticizzante. I campioni rivestiti erano più scuri ed avevano un colore giallo più intenso quando comparati al controllo. Si osservava inoltre un significativo decremento della forza di taglio dei campioni rivestiti fritti, che stava ad indicare una diminuzione della durezza di questi campioni.

INTRODUCTION

Deep-fat frying is a popular method of food preparation because of the taste and texture of fried products. The high temperature processing and high lipid content of lipids that comes from frying results in a dry food that has a crispy crust and soft inside. Typical examples are french-fries and nuggets which are popular in the fast food business. Nevertheless, consumers demand products with a low fat content, because lipid consumption is considered to be a major dietary contributor to high blood cholesterol and high blood pressure, both of which are associated with cardiovascular diseases (ALBERT and MITTAL, 2002).

Normally, hydrocolloids are added to food products in order to control and improve functional properties such as viscosity, water-holding capacity and emulsification. Some hydrocolloids, mainly polysaccharides, can be applied as edible coatings with the aim of lowering oil uptake during deep-fat frying (WILLIAMS and MITTAL, 1999; ALBERT and MITTAL, 2002). Coatings based on cellulose derivatives are used the most to provide a barrier to oil absorption during the frying process (ALBERT and MITTAL, 2002; GARCIA *et al.*, 2002, 2004; RIMAC-BRNCIC *et al.*, 2004). Another applicable hydrocolloid is cassava starch which is a cheap and abundant raw material (VICENTINI *et al.*, 2005). To date this colloid has not been used in studies on oil uptake during frying. The aim of this study was to apply edible coatings based on cassava starch and methylcellulose to reduce oil uptake in chicken nuggets during deep-fat frying.

MATERIALS AND METHODS

The coatings were prepared from solutions containing 4 g of cassava starch (CS) (VICENTINI *et al.*, 2005) or 1 g of methylcellulose (MC) (GARCIA *et al.*, 2002) per 100 g of solution. CS was donated by Flor de Lotus Co. (Botucatu - SP, Brazil), and MC was provided by Dow Chemical (Torrance, CA, USA). Glycerol (Synth S.A., São Paulo, Brazil) was used as a plasticizer at 25 g and 55 g glycerol/100 g biopolymer.

The chicken nuggets were prepared according to TRINDADE *et al.* (2001), in a single batch. The coatings were applied by dipping the nuggets in the coating suspensions at 40°C, for 5 sec. After that, the nuggets were breaded (Batistela Alimentos, São Paulo, Brazil), pre-fried at 180°C for 30 seconds and stored in a freezer, at -18°C. The frozen pre-fried samples were later fried at 180°C for 3 minutes, without previous thawing. Nuggets without coatings were also prepared and tested under the same conditions as the coated nuggets. Commercial hydrogenated vegetable oil (Bunge Alimentos, São Paulo, Brazil) was used in the pre-frying and frying process. Fresh oil was always used for pre-frying and frying.

Pre-fried and fried samples were analyzed to determine the water and lipid contents, in four replicates obtained from one frying batch. For these analyses, the samples consisted of the surface layer (coating + breading), defined as crust, the internal portion of the nugget and the whole nugget. The crust was removed using a scalpel after careful visual inspection. Water content (WC) was determined by drying in an oven at 105°C for 16 h. The lipid content (LC) was determined, on previously dried samples, by Soxhlet extraction using chloroform.

After the WC and LC were determined, the relative increase of water retention (WR) and oil uptake decrease (OU) in coated samples were calculated according to GARCIA *et al.* (2002):

$$WR = (WC_{coated}/WC_{control} - 1) \times 100 \quad (1)$$

$$OU = (1 - LC_{coated}/LC_{control}) \times 100 \quad (2)$$

where WC and LC are the water content and lipid content of the samples, respectively (coated or control, i.e., without coating) on the defatted dry basis.

Whole fried nuggets were also analyzed to determine the color of the surface, and the shear and compression forces, at room temperature (25°C). The shear force was also determined on the internal portion of the nuggets.

Colorimetric measurements were carried out with a portable colorimeter (mod. Miniscan XE, HunterLab, Reston, VI, USA). The color parameters are expressed using the color standards of the CIELab – "Comission Internationate de L'Eclairage" system: lightness (L*) and chromaticity parameters a* (red-green) and b* (yellow-blue). Triplicate readings were carried out at three different locations on each sample.

For texture characterization, a texture analyser TA.XT2*i* (SMS, Surrey, UK) was used. Whole nuggets were sheared using the knife blade probe; cylindrical samples (20 mm diameter) were compressed with a cylindrical probe (20 mm diameter) until 50% deformation. The internal portion (1 cm width x 2 cm length) of nuggets was sheared using a Warner-Bratzler probe. In all these tests, done in triplicate, the probes moved at 500 mm/min.

The results were submitted to analysis of variance (ANOVA) and the Duncan mean comparison test. The significance level used was 0.05 (SAS, 1989). The treatments were: 1) Control: nuggets without edible coatings; 2) CS 25: nuggets coated with 4% cassava starch in solution and 25 g glycerol/100 g cassava; 3) CS 55: nuggets coated with 4% cassava starch in solution and 55 g glycerol/100 g cassava; 4) MC 25: nuggets coated with 1% methylcellulose in solution and 25 g glycerol/100 g methylcellu-

Table 1 - Oil uptake reduction* and water retention* of the crust, internal portion and whole nuggets coated with: CS-cassava starch (4 g CS/100 g of solution), and MC-methylcellulose (1 g MC/100 g of solution), with 25 (CS 25 or MS 25) and 55 g (CS 55 or MC 55) of glycerol/100 g of biopolymer after frying.

	Sample/Treatment	CS 25	CS 55	MC 25	MC 55
Oil Uptake	Crust	18.8±2.4^{a}	21.8±7.0^{a}	7.8±4.3^{b}	13.0±4.6^{ab}
	Int. Portion	-9.2±1.0^{a}	-11.1±11.4^{a}	-3.8±16.7^{a}	-0.6±10.0^{a}
	Whole Nuggets	16.3±1.2^{a}	7.4±4.3^{bc}	6.7±1.9^{b}	11.1±1.4^{c}
Water Retention	Crust	26.1±9.6^{ab}	47.9±16.7^{a}	23.7±12.1^{ab}	19.3±8.6^{b}
	Int. Portion	8.0±3.5^{a}	6.2±4.7^{a}	10.0±2.3^{a}	5.1±3.4^{a}
	Whole Nuggets	-1.7±3.3^{a}	3.1±10.4^{a}	-3.6±0.8^{a}	-8.9±6.0^{a}

$^{a-d}$ Means in the same line with different superscripts are significantly different ($p \leq 0.05$).
* Relative to the control, according to Equations 1 and 2.

lose; and 5) MC 55: nuggets coated with 1% methylcellulose in solution and 55 g glycerol/100 g methylcellulose.

RESULTS AND CONCLUSIONS

All coating treatments reduced oil uptake in relation to the control (Table 1); all the nuggets had a lower lipid content than the control (Fig. 1), after frying. The lowest lipid content was found in the whole nuggets coated with cassava starch (CS) with 25% plasticizer; the oil uptake was reduced 16% in relation to the control (Table 1). The coating treatments however did not significantly affect the water retention and consequently, no significant difference was observed in the water content of fried whole nuggets (Fig. 1).

The highest oil uptake reduction (18%) after pre-frying was observed in nuggets coated with methylcellulose (MC) with 55% of plasticizer. Regarding the lipid content of pre-fried nuggets, no significant difference was observed between the nuggets coated with MC and the two plasticizer concentrations; this was probably due to a high standard deviation in both cases. Likewise, no effect was observed concerning the water content of pre-fried nuggets (Fig. 1).

The relatively high lipid content values in the fried nuggets (30-39% defatted dry basis, ddb) were due to the high lipid content of the crust of the nuggets (Fig. 1), which was not protected by hydrocolloid coatings, because the coatings were applied to the nuggets before breading. Similar behavior was observed by HOLOWNIA *et al.* (2000) working with edible films based on methylcellulose and hydroxypropylmethylcellulose applied to marinated whole chicken strips before breading. Although the crusts were not protected, the different lipid content values were probably due to the water migration from the hydrocolloids towards the crust, which compromised the oil uptake during frying, in certain cases. Practically, during the frying process the moisture is replaced by the oil; it is a dehydration process (SAGUY *et al.*, 1998). It is also possible that the hydrophilic properties of the hydrocolloids repelled the oil adjacent to the coating and thus lowered the overall lipid content of the crust. These phenomena could increase the water-holding capacity of the coating by entrapping the moisture and consequently, preventing oil uptake. The values obtained after the pre-frying process could be of interest to the meat product industry, because, in nugget processing, pre-frying

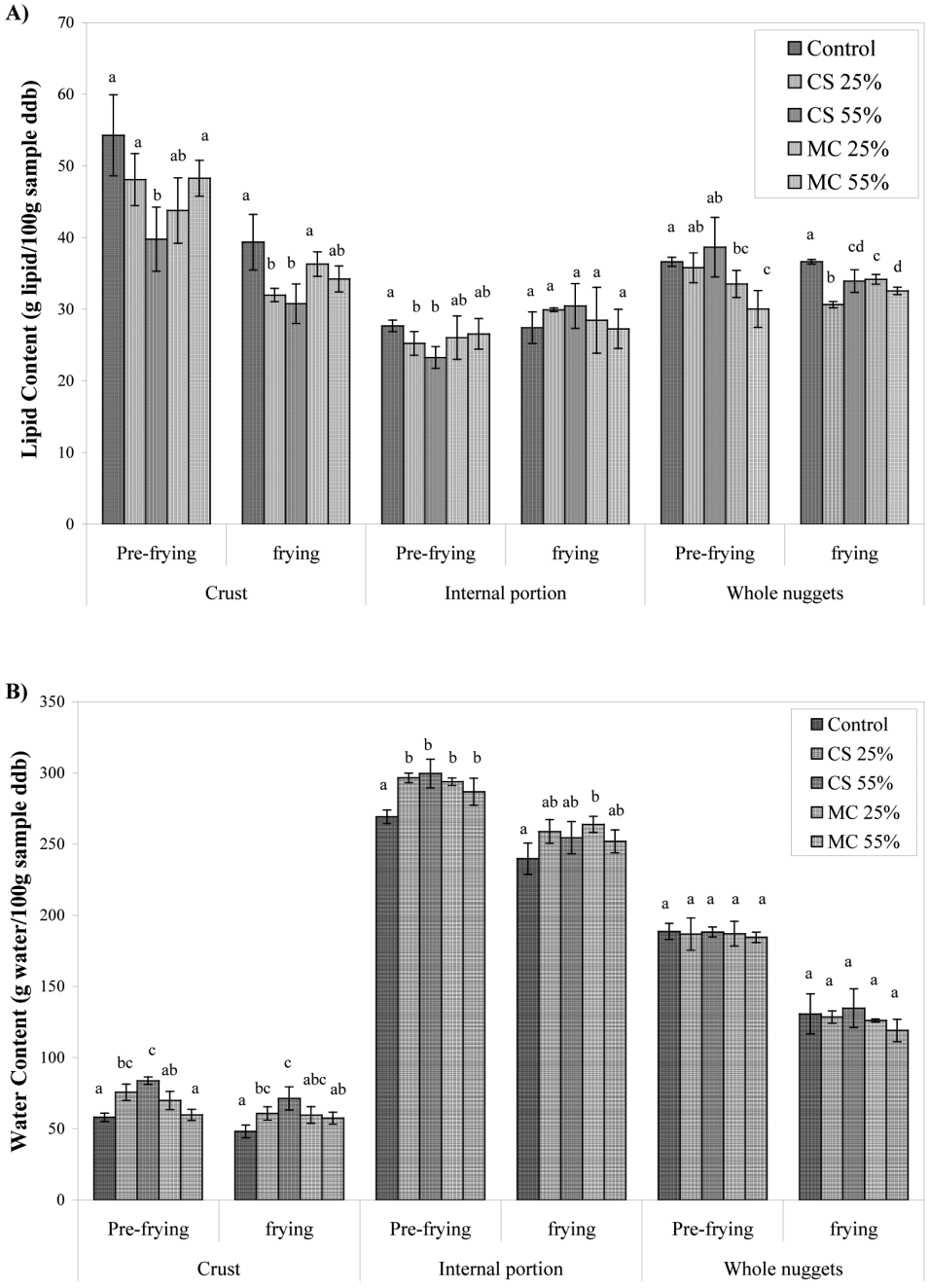


Fig. 1 - Lipid (A) and water (B) contents (g/100 g sample, ddb = defatted dry basis) of coated nuggets and control (uncoated), determined in the crust, internal portion and whole nuggets. Coating formulations: CS-cassava starch (4 g CS/100 g of solution), and MC-methylcellulose (1 g MC/100 g of solution), with 25 and 55 g de glycerol/100 g of biopolymer. [a-d] Means within a group with different superscripts are significantly different ($p \leq 0.05$).

is an important step for the adherence of the breading, color development and crispness.

The internal portion of the nuggets had a relatively low lipid content during frying (~29%) as well as during pre-frying, without effect of treatments. But, in this case, there were significant differences with respect to the CS coatings.

Cellulose-based coatings have been used in deep-fat fried products such as biscuits prepared with a commercial mixture (ALBERT and MITTAL, 2002), potatoes (GARCIA *et al.*, 2002, 2004), and pastry mix (WILLIAMS and MITTAL, 1999), where the protective effect of coatings is evident, in contrast to what was observed in this work. A possible explanation for why the coatings in this work were not effective is that the lipids were mainly absorbed by the breading material, but the possibility of the loss of the coating integrity cannot be discarded. This behavior however is complex, because the degree of oil uptake during deep-fat frying is significantly affected by many factors such as process conditions (temperature, time), pre-treatment, the physical-chemical characteristics of the food and the origin and composition of the oil (RIMAC-BRNCIC *et al.*, 2004). According to HERSHKO and NUSSINOVITCH (1998), the chemical similarity between coating solutions and the product surface, as well as the properties of the surface are important for the successfull adhesion of the coating.

Surface color parameters (L*, a*, b*) of the fried coated nuggets were slightly darker than the control, that is, they had lower L* values, and a yellow coloring (high a* values) without a clear effect of any of the coatings (Table 2). From a technological point of view, this could be of interest because the consumer would not notice any difference due to the presence of these coatings.

Another technological aspect was the effect of coating on the textural properties of the coated nuggets. In general, the coated nuggets, independent of the treatment, were softer than the control. In contrast the shearing force of the coated samples varied from 57 to 70 N, while the control samples had higher values, near 78 N (Table 2). These differences were even higher in the compression tests; the controls showed compression force values of 40 N, while these values varied from 20 to 27 N in the coated samples.

ACKNOWLEDGMENTS

The authors wish to thank the CYTED (Project XI.20) and FAPESP for the IC fellowship of MRM (04/07083-0).

A form of this paper was presented at the SLIM06 "Shelf Life International Meeting" held in Catania, Italy, June 21-23, 2006.

Table 2 - Surface color parameters (L* a* b*), shearing force (Fsh) and compression force at 50% (F_{Comp}) of whole nuggets, and shearing force (Fsh_{int}) of the internal portion of the coated nuggets with: CS-cassava starch (4 g CS/100 g of solution) and MC-methylcellulose (1 g MC/100 g of solution) with 25 and 55 g of glycerol/100 g of biopolymer.

	Properties	Control	CS 25	CS 55	MC 25	MC 55
Color	L*	62.7±0.8^{a}	56.2±2.3^{b}	57.9±2.8^{bc}	57.8±1.3^{bc}	60.0±1.5^{c}
	a*	10.5±0.7^{a}	13.9±2.0^{c}	12.5±1.0^{b}	11.8±0.9^{ab}	11.2±0.5^{ab}
	b*	40.5±3.6^{a}	39.5±3.8^{a}	40.5±2.9^{a}	40.1±1.9^{a}	40.1±2.7^{a}
Texture	Fsh (N)	77.8±11.3^{a}	57.8±9.6^{b}	69.6±7.0^{ab}	60.4±5.4^{b}	65.1±7.5^{ab}
	Fsh_{int} (N)	8.6±1.4^{a}	8.6±1.8^{a}	7.8±0.9^{a}	7.6±1.6^{a}	7.7±1.5^{a}
	F_{Comp} (N)	39.6±5.1^{a}	23.1±4.1^{b}	24.4 ± 9.9^{b}	26.56± 9.0^{b}	19.8±6.5^{b}

$^{a-d}$ Means in the same line with different superscripts are significantly different ($p \leq 0.05$).

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Revised paper received June 11, 2007 Accepted July 30, 2007

SHORT COMMUNICATION

SOLID-PHASE MICROEXTRACTION IN THE ANALYSIS OF THE VOLATILE FRACTION OF EXTRA-VIRGIN OLIVE OIL FROM FOURTEEN CULTIVARS GROWN IN THE LAKE GARDA REGION (ITALY)

SULLA FRAZIONE VOLATILE DI QUATTORDICI OLI EXTRA-VERGINI DI OLIVA PRODOTTI DA CULTIVARS DEL LAGO DI GARDA

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ABSTRACT

Forty-one volatile compounds were identified in virgin olive oils from fourteen cultivars grown near Lake Garda, Italy, using headspace solid-phase microextraction coupled with gas chromatography/mass spectrometry. Analyses of oils from olives with comparable ripeness indices showed that C6 volatile compounds, enzymatically produced through the lipoxygenase pathway, accounted for 77.4-94.3% of the total volatile fraction. The C5 volatile compounds, which are not derived from the lipoxygenase pathway, ac-

RIASSUNTO

In questo lavoro gli Autori riportano i risultati ottenuti dalla valutazione dello spazio di testa della frazione volatile di olio extra vergine di oliva ottenuto da 14 cultivars differenti impiantate su terreno situato a sud-ovest del Lago di Garda. Le analisi effettuate hanno mostrato che i composti volatili C6 prodotti attraverso la via della lipossigenasi, per oli prodotti da olive con valore comparabile dell'indice di maturazione, rappresentano 77,4-94,3% della frazione volatile: viene inoltre riportata la distribuzione di questi composti volatili.

- Key words: headspace solid-phase microextraction, Lake Garda oils, virgin olive oil, volatile compounds -

counted for 1.6-8.4% of the total volatile fraction.

I composti volatili C5, che non derivano dalla via lipossigenasi, rappresentano 1,6-8,4% della frazione volatile.

INTRODUCTION

In the Lombard area, most olive trees are grown along the shores of Lakes Garda, Iseo, and Como. Over the past 15 years, the level of oil production has increased from 300 tons/year to almost 700 tons/year (PEDO' *et al.*, 2003). Extra-virgin olive oil from the Lake Garda Riviera has recently become an important niche product.

Various authors (ANGEROSA *et al.*, 1999; ANGEROSA, 2002; BENINCASA *et al.*, 2003; OLIAS *et al.*, 1993) have reported that the lipoxygenase (LOX) pathway is responsible for production of the most abundant volatile compounds found during the oil extraction process and the enzymatic activity of this pathway is known to be related to agronomic and technological factors. The influence of environmental conditions on the production of volatile compounds has also been demonstrated (APARICIO and MORALES, 1998; VICHI *et al.*, 2003; WILLIAMS *et al.*, 1998).

Many analytical procedures have been used to identify and quantify the volatile compounds that characterize olive oil aroma. Among the extraction techniques, headspace solid-phase microextraction (HS-SPME) is the simplest technique for differentiation studies based on profile comparison. Numerous studies have been reported on characterization of the volatile fraction of olive oil (ANGEROSA *et al.*, 1999, 2004; ANGEROSA, 2002; BACCOURI *et al.*, 2007; CAVALLI *et al.*, 2004; DHIFI *et al.*, 2005; HADDADA *et al.*, 2007; JIMENEZ *et al.*, 2004; LUNA *et al.*, 2006; MILDNER-SZKUDLARS *et al.*, 2003; MONTEDORO and SERVILI, 1992; TURA *et al.*, 2002, 2004; VICHI *et al.*, 2003).

The aim of this study was to obtain initial information concerning the volatile compound fraction of olive oils derived from local cultivars growing in the Lake Garda region of Italy.

MATERIALS AND METHODS

Samples

Olives were harvested from fourteen cultivars (Regina, Miniol 2, Trepp, Less, Baia, Pendolino, Maurino, Grignano, Gargnà, Casaliva, Rossanello, Mitria, Favarol 1, Leccino) that were growing in an 8,000 m^2 experimental plot at an altitude of 140-215 m asl near the village of Raffa di Puegnago southwest of Lake Garda. The olives were all cultivated under the same conditions and were harvested within the month of November, 2003. Each individual cultivar was stored separately for no longer than one week in open plastic containers with a 10 kg capacity at 7±2°C to simulate real storage conditions.

The ripeness indices were determined according to the indications of the Estación de Olivicultura y Elaiotecnia, Jaén (HERMOSO *et al.*, 1991). Oil was extracted separately from each of the fourteen cultivar using a standard discontinuous procedure. In total, 18 kg of olives per cultivar were ground with an Inox hammer crusher and malaxed at 28°C for 30 min. The oil was extracted using a hydraulic press (maximum 200 bars) and separated by centrifugation at 2,000 rpm.

Three 10 mL aliquots of each oil were taken as samples, centrifuged at 5,000 rpm for 10 min, then stored for two

months at 10°C in the dark and analyzed within 10 days. Three series of fourteen analyses were performed. The data reported in Table 1 are the means of three series of analyses. The oils were classified on the basis of acidity, peroxide index, UV light absorption (K_{232} and K_{270}), and fatty acid and sterol content, following the analytical methods described in Regulations EEC/2568/91 and EEC/1429/92 of the European Union Commission. All the samples were categorized as extra virgin olive oil according to the European Commission EU Regulation 796/2002 (EU 2002), which was in force at the time of the experiment.

Analysis of volatile compounds

Each olive oil sample (0.2 kg derived from approximately 2.5 kg) was spiked with an internal standard (4-methyl-2-pentanol; Sigma Aldrich, Milan, Italy) to a concentration of 1.0 μg/g. Three 5-g aliquots of each sample were weighed and placed in a 40-mL headspace vial fitted with a Teflon-lined septum. The sample was placed in a water bath at 40°C with a magnetic stirrer. After 15 min, an SPME fiber covered with 2 cm of 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (Supelco, Milan, Italy) was inserted into the headspace of the vial for 60 min. The volatile compounds were desorbed for 10 min at 220°C in a gas chromatograph with a split/splitless injection port. The injector operated in split mode (1:5). Gas chromatography/mass spectrometry (GC/MS) analyses were performed in a Shimadzu 2010 gas chromatograph coupled to a Shimadzu QP-2010 MSD quadrupole mass spectrometer (Shimadzu, Milan, Italy). The gas chromatograph was equipped with an Equity™-5 column (30 m x 0.25 mm internal diameter x 0.25 μm film thickness, Supelco, Milan, Italy).

The operating conditions for the GC/MS were as follows: helium flow, 1.0 mL/min; oven temperature 50°C for 1 min increased to 240°C at a rate of 3°C/min. The temperature of the ion source was 200°C, the electron energy was 70 eV, and the interface temperature was 260°C. Mass spectra were acquired over the mass range 40-300 amu.

Before sampling, the fiber was reconditioned for 10 min in the GC injection port at 250°C, and blank runs were carried out periodically during the study. The efficiency of the fiber was periodically verified by monitoring the signal of the internal standard.

Volatile compounds in the headspace of the virgin olive oils under study were identified by matching their mass spectra with the reference mass spectra of a private library and the NIST 147 library.

Quantitative analysis was carried out using the internal standard method. The concentration of the main LOX pathway volatiles was calculated using relative experimental response factors. The remaining compounds were quantified by considering the relative response factor to be 1. For compounds that were not completely resolved, a quantitative evaluation was performed using single or multiple ions for each peak and adopting specific factors derived from mass spectra. This information is useful for calculating the TIC (Total Ion Chromatogram) signal from the SIM (Single Ion Monitoring) signal.

RESULTS AND DISCUSSION

Ripeness indices and data from the HS-SPME analysis characterizing the volatile compounds present in the fourteen virgin olive oils are shown in Table 1. The ripeness indices of eleven of the cultivars ranged from 2 to 3. The other three ripening indices were 1.54 for the cultivar Rossanello, 4.19 for the cultivar Baia and 5.08 for the cultivar Leccino. These exceptions were due to the difficulty of har-

Table 1 - Volatile compounds (mg/kg ± standard deviation) in fourteen virgin olive oils from different cultivars grown on a single plot in the Lake Garda region.
n.d.: compound not detected, tr: trace (<0.01 mg/kg); [a]RI, Kovats retention index.

	RI[a]	Regina	Miniol 2	Trepp	Less	Baia
ethanol	554	0.38 ±0.06	0.11±0.03	0.15±0.03	0.05±0.02	1.02±0.10
acetic acid	620	0.51±0.07	0.31±0.05	0.28±0.06	0.25±0.06	0.49±0.07
3-methyl butanal + 2-methyl butanal	638	0.11±0.03	0.05±0.02	0.07±0.02	0.04±0.01	0.07±0.02
1-penten-3-ol	663	0.80±0.10	0.43±0.07	0.20±0.05	0.15±0.03	0.40±0.07
1-penten-3-one	673	1.19±0.11	0.49±0.07	0.27±0.06	0.19±0.03	0.29±0.06
pentan-3-one	677	0.54±0.07	0.11±0.02	0.06±0.02	0.03±0.02	0.96±0.12
pentanal	700	0.05±0.02	0.09±0.02	0.05±0.02	0.08±0.03	0.15±0.03
3-methyl-butan-1-ol + 2-methyl-butan-1-ol	722	0.06±0.01	0.07±0.01	tr.	tr.	0.08±0.01
(E)-2-pentenal	724	0.20±0.06	0.07±0.01	0.03±0.01	tr.	0.10±0.04
pentan-1-ol	741	0.13±0.02	0.02±0.01	tr.	n.d.	0.16±0.03
toluene	743	0.14±0.02	0.11±0.03	0.10±0.03	0.23±0.06	0.38±0.07
(Z)-2-penten-1-ol	747	1.17±0.10	1.23±0.10	0.59±0.07	0.38±0.06	1.64±0.10
(Z)-3-hexenal	774	15.84±0.92	16.53±1.20	0.24±0.06	0.34±0.07	4.36±0.32
hexanal	777	1.52±0.12	0.79±0.10	1.38±0.10	0.82±0.10	1.18±0.10
(E)-2-hexenal	827	8.84±0.70	4.18±0.12	45.00±1.30	43.25±1.30	5.83±0.50
(Z)-3-hexen-1-ol	834	11.31±1.05	11.88±0.05	0.86±0.05	2.41±0.14	15.02±1.10
(E)-2-hexen-1-ol	849	1.22±0.12	0.10±0.03	0.82±0.05	0.82±0.05	1.58±0.12
hexanol	854	1.23±0.12	0.20±0.01	0.21±0.03	0.20±0.03	2.71±0.20
p-xilene	868	0.16±0.02	0.10±0.03	0.10±0.03	0.25±0.03	0.26±0.03
styrene	871	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.05±0.01
heptan-2-one	874	0.47±0.06	0.40±0.06	0.04±0.01	0.02±0.01	0.26±0.03
heptanal	883	0.07±0.01	0.06±0.01	0.06±0.01	0.13±0.02	0.14±0.02
(E.E)-2.4-hexadienal	887	0.17±0.03	0.10±0.02	0.12±0.02	tr.	0.12±0.02
3-ethyl-1.5-octadiene (E or Z)	907	0.26±0.02	0.18±0.02	0.06±0.01	0.02±0.01	0.21±0.02
3-ethyl-1.5-octadiene (E or Z)	912	0.84±0.10	0.48±0.06	0.35±0.05	0.20±0.03	0.92±0.10
(E)-2-heptenal	931	1.76±0.10	1.10±0.10	0.67±0.04	0.42±0.06	1.83±0.10
5-ethyl-2(5H) furanone	940	n.d.	n.d.	0.04±0.01	tr.	0.07±0.01
heptan-1-ol	961	0.72±0.05	0.68±0.05	n.d.	n.d.	0.57±0.04
hexanoic acid	965	0.07±0.01	0.06±0.01	0.16±0.02	0.09±0.02	n.d.
6-methyl-5-hepten-2-one	970	n.d.	tr.	Tr.	n.d.	n.d.
octan-2-one	974	n.d.	n.d.	n.d.	n.d.	n.d.
octanal	981	0.05±0.01	0.06±0.01	0.15±0.02	0.03±0.01	n.d.
(Z)-3-hexenyl acetate	994	0.22±0.02	0.51±0.06	0.05±0.01	0.07±0.01	n.d.
hexyl acetate	999	n.d.	0.05±0.01	0.04±0.01	0.02±0.01	n.d.
p-cymene	1010	0.02±0.01	n.d.	tr.	n.d.	n.d.
limonene	1016	0.23±0.05	0.24±0.05	0.28±0.03	0.16±0.02	1.00±0.10
(E)-β-ocimene	1040	0.04±0.01	0.03±0.01	tr.	0.02±0.01	0.13±0.02
nonanal	1087	0.09±0.02	0.17±0.02	0.18±0.02	0.06±0.01	0.11±0.02
phenylethyl alcohol	1102	0.02±0.01	0.02±0.01	0.03±0.01	n.d.	0.05±0.01
α-copaene	1390	n.d.	n.d.	n.d.	n.d.	0.04±0.01
α-farnesene	1513	0.03±0.01	n.d.	n.d.	n.d.	n.d.
% C6/total volatile compounds		79.6	83.4	92.2	94.3	72.7
% C5/total volatile compounds		8.4	6.3	2.4	1.8	9.2
Degree of ripeness		2.56	2.38	2.72	2.40	4.19

Table 1 - Volatile compounds (mg/kg ± standard deviation) in fourteen virgin olive oils from different cultivars grown on a single plot in the Lake Garda region.
n.d.: compound not detected, tr: trace (<0.01 mg/kg); [a]RI, Kovats retention index.

Pendolino	Maurino	Grignano	Gargnà	Casaliva	Rossanello	Mitria	Favarol	Leccino
0.11±0.03	0.29±0.06	0.07±0.01	0.35±0.06	0.18±0.04	3.41±0.10	0.17±0.03	0.27±0.05	1.82±0.10
1.00±0.10	0.06±0.01	0.26±0.06	0.27±0.06	0.25±0.06	0.18±0.03	0.37±0.06	0.27±0.04	0.52±0.07
n.d.	n.d.	n.d.	0.18±0.03	0.10±0.03	0.04±0.01	0.24±0.05	0.10±0.03	0.07±0.02
0.24±0.05	0.43±0.07	0.33±0.06	0.34±0.06	0.31±0.06	0.55±0.10	0.51±0.08	0.24±0.06	0.15±0.03
0.35±0.06	1.11±0.10	0.49±0.07	0.24±0.06	0.31±0.06	0.44±0.07	0.55±0.05	0.17±0.03	0.13±0.03
0.12±0.03	0.15±0.03	0.09±0.03	0.15±0.02	0.06±0.02	0.26±0.03	0.20±0.06	0.02±0.01	0.03±0.01
0.19±0.02	0.15±0.06	0.11±0.02	0.22±0.06	0.18±0.06	0.20±0.05	0.17±0.04	0.27±0.05	0.04±0.01
tr.	tr.	0.03±0.01	0.05±0.01	0.03±0.01	0.14±0.03	0.08±0.01	0.02±0.01	0.09±0.01
0.06±0.01	0.19±0.03	0.07±0.01	0.05±0.01	0.03±0.01	0.06±0.01	0.06±0.01	0.03±0.01	0.06±0.01
n.d.	n.d.	n.d.	0.04±0.01	tr.	0.07±0.01	0.05±0.01	n.d.	n.d.
0.28±0.07	0.14±0.03	0.31±0.03	0.23±0.06	0.22±0.05	1.15±0.1	0.15±0.04	0.13±0.06	0.16±0.03
0.41±0.05	0.69±0.04	0.31±0.06	0.79±0.10	0.70±0.10	1.22±0.10	1.40±0.10	0.57±0.05	0.42±0.07
0.34±0.06	16.18±0.98	12.39±1.00	0.32±0.07	0.32±0.06	14.92±1.02	0.49±0.07	0.17±0.03	0.13±0.04
1.56±0.10	2.00±0.12	1.44±0.10	2.10±0.13	1.74±0.10	2.09±0.10	1.65±0.11	1.08±0.09	0.93±0.10
64.59±1.70	7.94±0.18	7.54±0.18	45.58±1.25	56.30±1.30	4.78±0.25	56.85±1.30	40.83±1.30	34.14±1.20
5.71±0.52	3.37±0.20	4.09±0.42	1.89±0.09	2.62±0.22	13.25±1.07	3.56±0.22	0.90±0.10	0.86±0.10
3.41±0.12	0.14±0.03	0.09±0.02	4.69±0.15	1.66±0.10	0.17±0.04	7.18±0.12	0.66±0.05	1.55±0.12
2.46±0.18	0.37±0.04	0.50±0.05	2.11±0.10	0.46±0.05	0.42±0.05	3.42±0.13	0.19±0.03	0.49±0.05
0.34±0.07	0.14±0.03	0.11±0.02	0.22±0.05	0.21±0.05	0.91±0.10	0.16±0.03	0.16±0.03	0.14±0.03
0.03±0.01	0.02±0.01	tr.	0.03±0.01	0.05±0.01	0.05±0.01	0.02±0.01	0.04±0.01	0.04±0.01
0.06±0.01	0.05±0.01	0.44±0.05	0.13±0.03	0.11±0.03	0.18±0.04	0.11±0.02	0.04±0.01	0.07±0.01
0.18±0.02	0.06±0.010	0.05±0.01	0.10±0.03	0.10±0.03	0.22±0.02	0.08±0.02	0.08±0.02	0.07±0.01
0.07±0.01	0.17±0.03	0.08±0.01	0.06±0.01	0.04±0.01	0.09±0.01	0.05±0.01	tr.	n.d.
0.06±0.01	0.06±0.01	0.19±0.03	tr.	tr.	0.21±0.02	0.13±0.03	tr.	0.15±0.03
0.39±0.05	0.92±0.10	0.32±0.04	0.26±0.03	0.32±0.05	0.27±0.03	0.46±0.04	0.20±0.03	0.14±0.03
0.97±0.10	2.51±0.18	0.97±0.10	0.38±0.06	0.49±0.05	0.46±0.05	0.71±0.08	0.34±0.05	0.26±0.03
0.04±0.01	0.08±0.01	0.03±0.01	n.d.	0.04±0.01	tr.	0.07±0.01	n.d.	0.02±0.01
0.14±0.02	0.55±0.05	0.56±0.05	n.d.	n.d.	0.14±0.02	0.04±0.01	n.d.	n.d.
0.04±0.01	0.09±0.02	n.d.	tr.	tr.	tr.	0.06±0.01	0.05±0.01	0.02±0.01
n.d.	0.04±0.01	0.02±0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.02±0.01	0.03±0.01	tr.	0.02±0.01	n.d.	0.03±0.01	0.02±0.01	tr.	tr.
0.05±0.01	0.04±0.01	0.02±0.01	0.05±0.01	0.03±0.01	0.06±0.01	0.04±0.01	0.04±0.01	0.02±0.01
0.06±0.01	0.03±0.01	0.42±0.06	0.07±0.01	0.15±0.02	0.26±0.03	n.d.	n.d.	n.d.
n.d.	n.d.	0.14±0.02	0.17±0.02	0.06±0.01	n.d.	n.d.	n.d.	n.d.
n.d.	0.02±0.01	0.02±0.01	tr.	n.d.	0.02±0.01	tr.	0.02±0.01	0.02±0.01
0.14±0.02	0.31±0.05	0.40±0.06	0.35±0.05	0.28±0.04	0.70±0.05	0.14±0.02	0.43±0.06	0.26±0.04
tr.	0.23±0.02	0.20±0.02	tr.	0.07±0.01	n.d.	tr.	tr.	0.28±0.02
0.11±0.02	0.21±0.02	0.17±0.02	0.16±0.02	0.05±0.01	0.05±0.01	0.06±0.01	0.10±0.02	0.06±0.01
0.02±0.01	0.04±0.01	0.05±0.01	tr.	0.02±0.01	0.04±0.01	0.09±0.02	0.08±0.02	0.03±0.01
n.d.	n.d.	0.03±0.01	n.d.	tr.	n.d.	n.d.	tr.	n.d.
n.d.	0.02±0.01	0.02±0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
93.5	77.4	82.2	92.3	93.7	76.2	92.2	92.2	88.2
1.6	7.0	4.4	3.3	2.6	6.4	4.1	3.0	2.3
2.91	2.98	2.67	1.97	2.95	1.54	1.91	2.77	5.08

vesting all of the cultivars with comparable ripeness values in one month. Data corresponding to the eleven oils derived from the olive cultivars with ripeness indices of 2-3 are presented below.

Forty-one volatile compounds were identified. The C6 and C5 compounds were quantified and the percentages calculated in relation to the total quantity of volatile compounds. The quantity of C6 volatile compounds ranged from 77.4 to 94.3%, and the quantity of C5 volatile compounds, not produced by the LOX pathway, ranged from 1.6 to 8.4%. The pentene dimers were not detected and are not included in the data reported as "C5 volatile compounds".

The data show a prevalence of compounds with linoleic acid (LA) and linolenic acid (LnA) precursors: hexanal, hexanol, and hexyl acetate from LA and (Z)-3-hexenal, (E)-2-hexenal, (E)-2-hexen-1-ol, (Z)-3-hexen-1-ol, and (Z)-3-hexenyl acetate from LnA. Higher levels of C5 volatile compounds were accompanied by lower levels of C6 compounds. The cultivars differed not only in terms of the level of the various C6 compounds, but also in terms of the level of various C5 compounds which contribute to olive oil aroma and flavor due to their well-defined sensory profiles and threshold levels (pentan-3-one, 1-penten-3-one, 1-penten-3-ol, pentan-1-ol, (Z)-2-penten-1-ol, 3- and 2-methyl butanal) (KALUA *et al.*, 2007) as well as others (phenylethyl alcohol and (E)-β-ocimene).

Levels of C6 aldehydes, alcohols and esters from the LOX pathway are shown in Table 2. Detailed data are reported for all volatile C6 compounds derived from the metabolism of 13-hydroperoxides of LA and LnA. Examination of the compounds derived from the LOX cascade shows that all samples contained higher levels of volatile compounds from LnA than those from LA, which is consistent with the literature (ANGEROSA *et al.*, 1999; ANGEROSA, 2002; BENINCASA *et al.*, 2003; OLIAS *et al.*, 1993).

Table 2 - C6 compounds (mg/kg) produced via the LOX pathway isolated from fourteen virgin olive oils from different cultivars grown on a single plot in the Lake Garda region.

	Regina	Miniol 2	Trepp	Less	Baia	Pendolino	Maurino	Grignano	Gargnà	Casaliva	Rossanello	Mitria	Favarol	Leccino
hexanal	1.52	0.79	1.38	0.82	1.18	1.56	2.00	1.44	2.10	1.74	2.09	1.65	1.08	0.93
hexanol	1.23	0.20	0.21	0.20	2.71	2.46	0.37	0.50	2.11	0.46	0.42	3.42	0.19	0.49
hexyl acetate	n.d.	0.05	0.04	0.02	n.d.	n.d.	n.d.	0.14	0.17	0.06	n.d.	n.d.	n.d.	n.d.
Σ C6 compounds from LA	2.75	1.03	1.64	1.05	3.89	4.02	2.37	2.08	4.38	2.26	2.51	5.07	1.27	1.42
(E)-2-hexenal	8.84	4.18	45.00	43.25	5.83	64.59	7.94	7.54	45.58	56.30	4.78	56.85	40.83	34.14
(Z)-3-hexenal	15.84	16.53	0.24	0.34	4.36	0.34	16.18	12.39	0.32	0.32	14.92	0.49	0.17	0.13
(E)-2-hexen-1-ol	1.22	0.10	0.82	0.82	1.58	3.41	0.14	0.09	4.69	1.66	0.17	7.18	0.66	1.55
(Z)-3-hexen-1-ol	11.31	11.88	0.86	2.41	15.02	5.71	3.37	4.09	1.89	2.62	13.25	3.56	0.90	0.86
(Z)-3-hexenyl acetate	0.22	0.51	0.05	0.07	n.d.	0.06	0.03	0.42	0.07	0.15	0.26	n.d.	n.d.	n.d.
Σ C6 compounds from LnA	37.42	33.19	46.97	46.88	26.78	74.10	27.66	24.53	52.55	61.06	33.38	68.08	42.56	36.69
% C6 aldehydes/Σ C6	65.2	62.8	95.9	92.7	37.1	85.1	87.0	80.3	84.3	92.2	60.7	80.6	96.0	92.4
% C6 alcohols/Σ C6	34.2	35.6	3.9	7.1	62.9	14.8	12.9	17.6	15.3	7.5	38.6	19.4	4.0	7.6
% C6 esters/Σ C6	0.5	1.6	0.2	0.2	0.0	0.1	0.1	2.1	0.4	0.3	0.7	0.0	0.0	0.0

These results allow the cultivars examined to be differentiated by several different criteria. The major components in the oil samples were aldehydes: the percent of C6 aldehydes/ ΣC6 ranged from 62.8% (Miniol cultivar) to 96.0% (Favarol 1 cultivar).

The olive oils produced from the Regina, Miniol 2, Maurino and Grignano cultivars showed non-negligible amounts of (Z)-3-hexenal (12.39-16.53 mg/kg) compared with the other olive oils. In these samples, (E)-2-hexenal was present in the range of 4.18-8.84 mg/kg. All other cultivar samples (Trepp, Less, Pendolino, Gargnà, Casaliva, Mitria and Favarol 1) contained higher levels of (E)-2-hexenal, ranging from 40.83 mg/kg to 64.59 mg/kg.

The percentage of alcohols, and to a lesser extent the percentage of esters, differed among the samples: the percent of C6 alcohols/ΣC6 was in the range of 4.0-35.6%. The olive oils from the Regina, Miniol 2, Maurino, and Grignano cultivars, containing a high level of (Z)-3-hexenal, had a (Z)-3-hexen-1-ol content ranging from 3.37 to 11.88 mg/kg.

The total quantity of aldehydes exceeded that of alcohols. The data in Table 2 suggest that (Z)-3-hexenal is not negligible for various cultivars. This finding contrasts with a previous study in which only a low level of this compound was detected (Guth and Grosch, 1993).

Other minor volatile compounds were observed in the virgin olive oils. The aldehydes heptanal, octanal, nonanal and (E)-2-heptenal were present in small quantities due to auto-oxidation reactions. Of the saturated aldehydes, hexanal was present in all samples at levels that did not exceed those found in high quality virgin olive oils.

Under the conditions adopted, the results show apparent differences among the varieties, both at the level of single volatile compounds and in the distribution of C6 volatile compounds derived from the metabolism of 13-hydroperoxides of LA and LnA. Considering the cultivars that had comparable ripeness indices, four (Regina, Miniol 2, Maurino and Grignano) showed that the predominant volatile compounds were (E)-2-hexenal, (Z)-3-hexenal, and (Z)-3-hexen-1-ol, with lower levels of hexanal, hexanol, (E)-2-hexen-1-ol, and (Z)-3-hexenyl acetate. In oils from seven cultivars (Trepp, Less, Pendolino, Gargnà, Casaliva, Mitria and Favarol 1), the predominant volatile compounds were (E)-2-hexenal and (Z)-3-hexen-1-ol, with lower levels of hexanal, hexanol, (Z)-3-hexenal, (E)-2-hexen-1-ol, and (Z)-3-hexenyl acetate. These two groups of cultivars differed mainly with respect to the total content of C6 aldehydes and C6 alcohols. The content of C5 compounds was higher for the first group of cultivars. Our data show that all the cultivars can be differentiated and the volatile compound composition is sufficiently distinct.

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Revised paper received June 11, 2007 Accepted August 7, 2007

A STUDY ON ORGANIC ACID, SUGAR AND KETONE CONTENTS IN TYPICAL PIEDMONT CHEESES

INDAGINE SUL CONTENUTO IN ACIDI ORGANICI, ZUCCHERI E CHETONI DI FORMAGGI TIPICI PIEMONTESI

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ABSTRACT

The dairy industry is a very important sector for the Piedmont (Italy) economy with ten Protected Denomination of Origin (PDO) cheeses and fifty-five classified as "Traditional". Among these "Traditional" cheeses, some are very interesting because of the quantity produced, cheese-making procedures used and sensory characteristics. Therefore, these cheeses might qualify for the PDO designation, but chemical information is lacking and only their gross composition is usually known.

RIASSUNTO

Il settore lattiero caseario costituisce per il Piemonte uno dei principali comparti produttivi e dei 33 formaggi DOP italiani ben dieci interessano il Piemonte e 55 sono quelli tradizionali. Fra questi ultimi alcuni per quantità prodotte o per caratteristiche tecnologiche, compositive e sensoriali risultano di estremo interesse e quindi potenzialmente soggetti alla attribuzione di una futura DOP, ma le conoscenze sulla loro caratteristiche compositive si limitano in genere esclusivamente ai macrocom-

- Key words: acetoin, cheese, diacetyl, organic acid, Piedmont cheeses, sugar -

The aim of this study was to characterise some Piedmont cheeses by studying important compounds responsible for their flavour. Sugars (lactose, glucose and galactose), organic acids (citric, orotic, pyruvic, lactic, oxalic, hippuric, formic, acetic, propionic, butyric, isobutyric, valeric and isovaleric) and ketones (diacetyl and acetoin) were quantitatively determined in 460 samples from 17 cheese varieties using a simple isocratic HPLC method. Lactose concentrations varied from 0 to 73.9 mg/g of cheese and were inversely correlated to ripening time. Glucose concentrations ranged from 0 to 4.3 mg/g of cheese while galactose showed a maximum value of 9.4 mg/g of cheese. The range of diacetyl and acetoin were from 0 to 4.3 mg/g and from 0 to 0.2 mg/ of cheese, respectively. Among the organic acids, lactic acid was the most abundant (0.3 to 39 mg/g). Citric acid was also very important with a maximum value of 4 mg/g. High variability characterised the carboxylic acids due to differences in raw material, cheesemaking technique and use of a starter. For all compounds high variability was observed due to home-made production, but nevertheless it was possible to obtain a good characterisation of all these cheeses.

I risultati di uno studio di caratterizzazione compositiva volto a valutare in 460 campioni di 17 formaggi piemontesi il contenuto di alcuni componenti minori quali zuccheri (lattosio, glucosio, galattosio), acidi organici (citrico, orotico, piruvico, lattico, ossalico, ippurico, formico, acetico, propionico, butirrico, isobutirrico, valerico ed isovalerico) e chetoni (diacetile ed acetoino) separati mediante HPLC. Il contenuto in lattosio è risultato compreso fra 0 e 73,9 mg/g di formaggio ed inversamente correlato al tempo di stagionatura. La concentrazione di glucosio è risultata compresa fra 0 e 4,3 mg/g per il galattosio mentre il valore massimo è risultato di 9,4 mg/g. La concentrazione di diacetile e di acetoino sono risultate comprese fra 0 e 4,3 mg/g e fra 0 e 0,2 mg/g rispettivamente. Fra gli acidi l'acido lattico risulta il più abbondante (0,3-39 mg/g). Molto importante altresì l'acido citrico con un massimo di 4 mg/g. Molto elevata per tutti i prodotti la variabilità in relazione alla artigianalità delle produzioni benché una loro discriminazione sia risultata comunque possibile.

INTRODUCTION

In the Piedmont region (northwestern Italy) with its vast area of land available for pasture, dairy farming and cheesemaking have become highly developed and are of great economic and cultural importance.

Of the thirty-three Italian cheeses with the Protected Denomination of Origin (PDO) according to European Regulation 510/06 (European Union, 2006), ten are produced in the Piedmont region and elsewhere, while seven (Bra duro, Bra tenero, Castelmagno, Robiola di Roccaverano, Murazzano, Toma Piemontese and Raschera) are produced exclusively in the Piedmont.

In addition to these PDO cheeses, about 55 cheeses, defined as "Typical" according to Italian law 350/99 (Decreto Presidente Repubblica, 1999), are also produced in Piedmont. While they are usually only available at local markets, there might be a way for farm cheesemakers to make a higher profit if they

are well-defined and their characteristics are better known. Unfortunately, information about the chemical characteristics of these cheeses is very scarce and only pertains to their gross composition (COISSON *et al.,* 2000; PATTONO *et al.,* 2001; ZEPPA *et al.,* 2003a, b; 2005).

Among the chemical, sensory and textural parameters, flavour profile is very important for cheese characterisation because it is strongly variety- or type-specific. This profile is influenced by many compounds, including organic acids, sulphur compounds, lactones or ketones, formed as a result of hydrolysis of milk fat during lipolysis, bacterial growth, normal ruminant metabolic processes and the addition of acidulants during cheesemaking (ADDA *et al.,* 1982; Fox *et al.,* 2004). Hence, quantitative determination of these compounds is especially important when studying flavour and nutritional qualities; they serve as indicators of microbial metabolism and as a classification index for cheese varieties (ADDA *et al.,* 1982; URBACH, 1993; FOX *et al.,* 2004).

The study focused on the following cheeses: Bra duro PDO, Bra tenero PDO, Castelmagno PDO, Cevrin di Coazze, Maccagno, Murazzano PDO, Murianengo, Plaisentif, Raschera PDO, Robiola d'Alba, Robiola di Roccaverano PDO, Saras del Fen, Toma del lait brusc, Tomino di Melle, Toma Piemontese PDO, Tomino di Talucco and Tuma 'd Trausela.

The Bra duro PDO is an uncooked, hard-cheese made with raw or pasteurized cow milk and ripened for almost 6 months. Starters are only used in the cheese made with pasteurized milk. The cheese is brine-salted; its cylindrical shape has a diameter of 30-40 cm and a height of 7-9 cm.

The Bra tenero PDO, Raschera PDO and Toma Piemontese PDO are uncooked semi-hard cheeses, made from raw or pasteurized cow milk and ripened for at least 2 months. Also for these cheeses, starters are only used in the cheeses made with pasteurized milk. Cheeses are brine- or dry salted. Toma Piemontese PDO and Bra tenero PDO are cylindrical (diameter of 30-40 cm and height of 7-15 cm) while Raschera PDO is square (side of 40 cm and height of 12-15 cm).

The Castelmagno PDO is a semi-fat, pressed, hard-paste, mould-inoculated cheese made from raw cow milk and a small quantity of ewe and/or goat milk and is ripened for 2-5 months. The production regulations prohibit the use of starters. The uniqueness of this cheese is that the curd is immersed in the whey for at least 48 hours. Its subsequent mincing in a special grinder and moulding imparts the characteristic very hard, granular and "chalky" consistency of this cheese. The cylindrical shape has a diameter of 15-25 cm and a height of 20-25 cm.

The Cevrin di Coazze cheese is a typical cheese that is produced by only five farmers. A mix of raw goat and cow milks is coagulated with liquid calf rennet at 40°-45°C. Starters are not used and the cheese is dry-salted. The cylindrical shape has a diameter of 15-18 cm and an height of 4-5 cm.

The Maccagno cheese is produced with raw cow milk coagulated with calf rennet at 35°-37°C. Starters are not used and the cheese is dry-salted. The cylindrical shape has a diameter of 18-20 cm and a height of 6-8 cm.

Murazzano PDO is a soft-cheese made from raw sheep milk or a mix of raw cow and sheep milk coagulated with liquid rennet and ripened for up to 10 days. The cylindrical shape has a diameter of 10-15 cm and a height of 3-4 cm.

Murianengo is a blue cheese produced in a small alpine area of Piedmont, near France, with raw cow milk coagulated with liquid calf rennet and ripened for almost 6 months. The milk is left to sour at room temperature for 12 hours then skimmed and added to the same quantity of fresh raw milk. Starters are not used and the cheese is dry-salted.

The cylindrical shape has a diameter of 20-25 cm, a height of 8-20 cm and weighs 8-10 kg.

Plaisentif is an ancient cheese produced with partially skimmed raw cow milk in a small mountain area in the Turin Province. The cylindrical shape has a diameter of 15-18 cm and a height of 4-5 cm. According to the regulations, it is only produced in June when the *Viola calcarata* L. flowers are present in the pastures. The milk is coagulated with liquid calf rennet at 30°-32°C. Starters are not used. The cheese is dry-salted.

The Robiola d'Alba and Robiola di Roccaverano PDO cheeses are similar to the Murazzano PDO cheese but are produced with pasteurized cow or sheep milk inoculated with starters and coagulated without rennet. All these cheeses are ripened for up to 3 days.

The Saras del Fen is a ripened ricotta cheese produced with a whey mix obtained from goat, sheep and cow milks. The whey is coagulated with citric acid and the product is ripened for at least 20 days. The cheese is spherical with a diameter of 12-15 cm and weight 500-800 g.

The Toma del lait brusc cheese is produced with naturally acidified cow milk. It is similar to Castelmagno PDO cheese but is only produced in some alpine areas. The cheese is very hard, granular and "chalky". The cylindrical shape has a diameter of 15-25 cm and a height of 20-25 cm.

Tomino di Melle is a fresh cheese (about 5 days of ripening) produced with fresh raw cow milk coagulated with liquid calf rennet at 30°-32°C. Starters are not used and the cheese is dry-salted. The cylindrical shape has a diameter of 10-15 cm and a height of 2-2.5 cm.

Tomino di Talucco is a fresh cheese produced with a mix of raw goat and cow milks. The cylindrical shape has a diameter of 10-12 cm and a height of 2-2.5 cm.

Tuma 'd Trausela is produced by only four farmers. It is a very fresh cheese without a crust. The milk is coagulated with calf rennet; the curd, without cutting, is drained for 8-10 hours before being sold.

The purpose of this study was to quantify the following flavour compounds: sugars, ketones and organic acids in these seventeen important Piedmont cheeses.

MATERIALS AND METHODS

Samples

Four-hundred and sixty samples from seventeen cheese varieties were collected and analyzed (Table 1). Samples were collected in 2005 from the cheese-makers of practically all of the cheese varieties. Due to the many cheese-makers who produce the Toma Piemontese PDO cheese only about 60% of the producers participated.

One or two cheeses from different batches were furnished directly by the producers. All samples were collected from a whole cheese ripened according to the specific cheese-making regulations (Table 1) and stored at -18°C after arrival in the laboratory.

Chemical analysis

Dry matter, fat and protein content were determined according to the Italian Official Methods for cheese analysis (D.M., 1986).

Organic acids (citric, orotic, pyruvic, lactic, oxalic, hippuric, formic, acetic, propionic, butyric, isobutyric, valeric and isovaleric), sugars (lactose, glucose and galactose), diacetyl and acetoin were determined by high performance liquid chromatography according to ZEPPA *et al.* (2001). Five grams of cheese were added to 25 mL of 0.013 N H_2SO_4 (mobile phase) and extracted for 10 min with a Stomacher blender (PBI,

Milano, Italy). The extract was subsequently centrifuged for 5 min (2,500×*g*) and the supernatant was filtered through a 0.20 μm disposable syringe membrane filter (Sartorius AG, Göttingen, Germany). The HPLC system (Thermo Electron Corporation, Waltham, MA, USA) was equipped with an isocratic pump (P1000), a multiple autosampler (AS3000) fitted with a 20 μL loop, a UV detector (UV100) set at 210 and 290 nm and a refractive index detector (RI-150). The detectors were connected in series. Data were collected on an EZChrom ver. 6.6 system (Thermo Electron Corporation, Waltham, MA, USA). The analyses were performed isocratically at 0.8 mL/min and 65°C with a 300x7.8 mm i.d. cation exchange column Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). Mobile phase was 0.013 N H_2SO_4 prepared by diluting reagent grade sulfuric acid with distilled water, filtered through a 0.45 μm membrane filter (Sartorius, AG, Göttingen, Germany) and degassed under vacuum. Each sample was analysed three times. Analytical grade reagents were used as standards (Sigma-Aldrich Corporation, Milano, Italy).

Statistical analysis

Conventional statistical methods were used to evaluate the chemical data. Analysis of variance and Duncan's multiple mean comparison test were applied using STATISTICA for Windows Release 7.1 (StatSoft Inc., Tulsa, OK, USA) to determine statistical differences between the cheese varieties.

RESULTS AND DISCUSSION

The gross composition of the 17 different varieties of cheeses is reported in Table 2. The concentration of organic acids, sugars and ketones is shown in Table 3. A great variability was observed within each cheese which was a consequence of differences in milk composition, starter cultures used, adventitious non-starter bacteria in milk, cheese-making and ripening conditions.

In spite of this variability the analysis

Table 1 - Identification code, milk generally used for cheese-making and ripening time for the cheeses.

Cheese variety	Number of samples	Identification code	Milk used	Ripening time (days)
Bra duro PDO	14	BD	Cow	90
Bra tenero PDO	12	BT	Cow	60
Castelmagno PDO	20	CA	Cow	60
Cevrin di Coazze	10	CE	Goat/Cow	60
Maccagno	16	MA	Cow	60
Murazzano PDO	18	MU	Sheep/Cow	10
Murianengo	12	MUR	Cow	90
Plaisentif	16	PL	Cow	90
Raschera PDO	19	RA	Cow	60
Robiola d'Alba	18	RdA	Cow	10
Robiola di Roccaverano PDO	44	RR	Sheep	10
Saras del Fen	24	SA	Goat/Sheep/Cow	20
Toma del lait brusc	23	TLB	Cow	90
Toma Piemontese PDO	174	TP	Cow	60
Tomino di Melle	12	TMELL	Cow	5
Tomino di Talucco	18	TAL	Goat/Cow	10
Tuma 'd Trausela	10	TRA	Cow	1

of variance showed significant differences among the tested cheese varieties for organic acids, sugars and ketones (Table 4). No significant differences among products were observed for orotic acid.

The mean values determined for these compounds are similar to those reported for similar products such as Pickled White cheese (AKALIN *et al.*, 2002), Greek cheeses (PAPADAKIS and POLYCHRONIADOU, 2005), Cheddar (KEEN and WALKER, 1974; LUES *et al.*, 1998), Canestrato Pugliese (FACCIA *et al.*, 2004), different goat cheeses (PARK and DRAKE, 2005; PARK and LEE, 2006), Feta (MANOLAKI *et al.*, 2006) and ricotta cheese (KINIK and GÜRSOY, 2004).

Lactose concentrations varied from 0 to 73.9 mg/g of cheese (Table 3) but lactose was absent in 207 samples. Only 61 of the analyzed cheeses had lactose concentrations that were higher than 1%. Lactose is metabolised by lactic acid bacteria (LAB) during cheesemaking and ripening at a rate that is largely determined by temperature and salt-in-moisture levels of curd (FOX *et al.*, 2004). Lactose was usually detected only in fresh unsalted cheeses such as Toma 'd Trausela, Tomino di Melle, Robiola d'Alba, Robiola di Roccaverano PDO, Tomino del Talucco and Saras del Fen and the quantity was higher in cheeses with shorter ripening times. Lactose was generally absent in all the other samples with longer ripening times. The concentrations of glucose and galactose, which are the two hydrolysis products of lactose, were also low. Glucose was absent in 309 samples since it is rapidly metabolized by LAB (FOX *et al.*, 2004) and the concentrations in twelve samples were about 0.1 mg/g of cheese. The maximum glucose concentration was about 4 mg/g in Saras del Fen cheese. The galactose concentration varied widely among samples with a maximum value of about 9.4 mg/g in Tomino del Talucco cheese. Some LAB can use galactose, but also the adventitious non-starter lactic acid bacteria have the potential to use this substrate. In fact a close relationship between the increase in this flora and

Table 2 - Basic composition (mean ± standard deviation) of the cheeses.

	Dry matter (%)	Protein (% dm)	Fat (% dm)	Ash (% dm)
Bra duro PDO	63.1±7.5	55.3±6.9	37.4±6.6	4.1±2.8
Bra tenero PDO	56.2±6.9	49.8±8.1	44.5±7.2	4.1±2.8
Castelmagno PDO	65.4±7.2	41.2±6.9	48.5±5.7	5.1±1.4
Cevrin di Coazze	48.4±4.6	48.7±8.8	31.4±7.2	7.6±1.7
Maccagno	53.1±4.6	43.5±3.1	46.5±5.7	4.1±2.1
Murazzano PDO	50.2±14.1	38.7±5.7	56.2±8.5	5.1±1.4
Murianengo	56.1±17.4	40.2±13.9	41.3±13.8	5.8±4.2
Plaisentif	57.1±2.9	45.2±3.7	47.3±4.6	7.5±1.7
Rashera PDO	55.6±7.2	48.5±7.6	48.7±6.8	4.3±2.1
Robiola d'Alba	44.3±4.6	36.1±5.2	49.8±9.4	3.4±0.8
Robiola di Roccaverano PDO	51.2±4.2	38.9±5.7	55.2±7.1	3.5±0.7
Saras del Fen	53.5±13.4	29.7±15.6	57.5±17.7	8.4±4.2
Toma del lait brusc	60.9±10.4	46.4±7.1	37.8±10.2	5.8±2.6
Toma Piemontese PDO	51.5±7.8	48.5±16.3	42.5±17.7	4.5±2.8
Tomino di Melle	52.4±6.4	39.5+7.8	47.9±9.5	4.1±3.1
Tomino di Talucco	40.8±1.1	47.1±1.9	42.1±2.5	5.7±0.7
Tuma 'd Trausela	37.3±2.2	48.4±2.6	38.8±3.5	5.1±0.3

(dm – dry matter).

Table 3 - Concentration (mg/g of cheese) of organic acids, sugars and ketones in the cheeses (m – minimum value; M – maximum value; SD – standard deviation). See Table 1 for cheese codes.

	BD				BT				CA			
	m	M	Mean	SD	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.323	1.426	0.835	0.352	0.045	0.886	0.361	0.262	0.193	1.822	0.737	0.386
Butyric acid	0.168	0.332	0.215	0.068	0.065	0.300	0.142	0.069	0.048	1.245	0.377	0.286
Citric acid	0.016	1.745	0.544	0.808	0.030	3.302	1.419	1.219	0.033	0.227	0.114	0.101
Formic acid	-	-	-	-	-	-	-	-	0.014	0.116	0.065	0.072
Hippuric acid	-	-	-	-	0.002	0.004	0.003	0.001	-	-	-	-
Iso-butyric acid	-	-	-	-	-	-	-	-	-	-	-	-
Iso-valeric acid	0.289	0.326	0.303	0.014	0.050	0.474	0.205	0.149	0.078	1.454	0.472	0.436
Lactic acid	19.146	39.099	26.311	6.729	9.177	28.327	17.934	4.946	5.120	24.452	14.832	3.870
n-Valeric acid	0.572	4.108	1.862	1.952	0.775	1.716	1.293	0.413	0.330	3.643	1.971	1.077
Orotic acid	0.001	0.020	0.012	0.007	0.003	0.036	0.021	0.011	0.000	0.020	0.012	0.006
Oxalic acid	0.309	0.336	0.322	0.014	0.035	0.210	0.129	0.072	0.127	0.155	0.140	0.012
Propionic acid	0.590	3.568	1.757	1.055	0.031	1.021	0.435	0.281	0.034	1.471	0.433	0.401
Pyruvic acid	0.006	0.195	0.117	0.071	0.007	0.050	0.025	0.012	0.002	0.257	0.093	0.083
Uric acid	0.000	0.003	0.001	0.001	0.001	0.004	0.003	0.001	0.000	0.004	0.001	0.001
Lactose	-	-	-	-	0.010	0.015	0.013	0.004	0.014	0.018	0.015	0.002
Galactose	0.002	0.057	0.025	0.029	0.015	7.158	2.894	2.634	0.002	0.098	0.037	0.043
Glucose	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin	0.004	0.008	0.006	0.001	0.001	0.027	0.009	0.008	0.002	0.010	0.007	0.003
Diacetyl	0.312	0.980	0.512	0.260	0.198	1.883	0.765	0.539	0.047	1.304	0.536	0.327

	CE				MA				MU			
	m	M	Mean	SD	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.050	0.450	0.283	0.196	0.017	0.638	0.390	0.198	0.038	1.248	0.352	0.507
Butyric acid	0.040	0.220	0.110	0.096	0.079	0.816	0.258	0.233	0.083	0.203	0.115	0.051
Citric acid	0.010	0.050	0.023	0.018	0.342	2.052	1.370	0.768	0.241	2.448	1.305	1.076
Formic acid	-	-	-	-	0.035	0.040	0.038	0.002	0.087	0.092	0.089	0.003
Hippuric acid	-	-	-	-	0.010	0.011	0.010	0.001	-	-	-	-
Iso-butyric acid	0.093	0.097	0.095	0.002	-	-	-	-	-	-	-	-
Iso-valeric acid	0.060	0.445	0.230	0.160	0.062	0.481	0.243	0.174	0.040	2.260	0.429	0.813
Lactic acid	2.895	21.105	11.743	8.632	14.895	25.001	19.965	3.131	20.301	30.621	24.618	3.841
n-Valeric acid	0.004	0.006	0.005	0.001	0.896	2.799	1.836	0.851	0.256	1.669	0.869	0.596
Orotic acid	0.005	0.020	0.015	0.007	0.001	0.038	0.011	0.013	0.000	0.029	0.016	0.010
Oxalic acid	0.010	0.175	0.064	0.076	0.071	0.268	0.163	0.078	0.149	0.638	0.307	0.153
Propionic acid	0.034	1.455	0.424	0.689	0.052	0.692	0.308	0.202	0.013	2.907	0.380	0.948
Pyruvic acid	0.005	0.055	0.024	0.019	0.005	0.058	0.035	0.017	0.030	0.493	0.157	0.163
Uric acid	0.001	0.002	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.004	0.002	0.001
Lactose	0.045	4.100	1.113	1.992	-	-	-	-	0.205	3.500	1.409	1.302
Galactose	0.147	0.307	0.227	0.113	0.020	6.357	2.423	3.072	0.145	2.141	0.769	0.862
Glucose	0.015	0.125	0.053	0.062	-	-	-	-	-	-	-	-
Acetoin	0.001	0.004	0.002	0.001	0.000	0.005	0.003	0.002	0.006	0.032	0.013	0.010
Diacetyl	0.225	3.470	1.178	1.303	0.633	1.798	1.124	0.461	0.208	0.690	0.409	0.179

Table 3 - (continued).

	MUR				PL				RA			
	m	M	Mean	SD	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.100	0.210	0.155	0.078	0.455	1.975	1.093	0.526	0.006	3.030	0.583	0.657
Butyric acid	0.140	0.410	0.274	0.131	0.135	0.925	0.513	0.235	0.106	0.302	0.177	0.061
Citric acid	0.005	0.070	0.047	0.036	0.025	0.870	0.444	0.338	0.200	2.244	1.322	0.720
Formic acid	-	-	-	-	0.150	2.991	0.681	0.945	0.158	0.580	0.369	0.298
Hippuric acid	-	-	-	-	-	-	-	-	-	-	-	-
Iso-butyric acid	-	-	-	-	0.025	0.130	0.048	0.041	-	-	-	-
Iso-valeric acid	0.390	1.005	0.794	0.282	0.025	0.445	0.235	0.138	0.072	0.744	0.236	0.186
Lactic acid	3.660	15.310	9.278	5.374	2.825	30.050	17.381	7.541	13.593	31.364	22.773	5.306
n-Valeric acid	0.003	0.007	0.005	0.002	0.145	3.035	0.855	1.228	0.401	2.003	1.252	0.772
Orotic acid	0.015	0.020	0.017	0.003	0.005	0.100	0.024	0.034	0.007	0.156	0.031	0.038
Oxalic acid	0.085	0.205	0.160	0.065	0.005	0.250	0.092	0.093	0.000	0.718	0.153	0.197
Propionic acid	0.064	0.770	0.472	0.366	0.677	3.896	1.411	1.013	0.065	1.813	0.473	0.481
Pyruvic acid	0.090	0.320	0.171	0.102	0.020	0.420	0.159	0.159	0.005	0.384	0.101	0.127
Uric acid	0.010	0.050	0.026	0.018	0.005	0.020	0.012	0.006	0.000	0.008	0.002	0.002
Lactose	0.005	0.015	0.010	0.007	1.572	1.596	1.581	0.011	0.090	0.180	0.135	0.064
Galactose	0.055	0.068	0.061	0.009	0.014	1.437	0.330	0.563	0.014	6.576	1.323	2.017
Glucose	0.005	0.125	0.053	0.052	0.020	0.645	0.333	0.442	0.015	0.025	0.020	0.005
Acetoin	0.001	0.010	0.006	0.005	0.005	0.210	0.058	0.102	0.003	0.017	0.009	0.005
Diacetyl	0.550	4.315	1.898	2.098	0.015	0.880	0.341	0.310	0.016	1.162	0.677	0.361

	RdA				RR				SA			
	m	M	Mean	SD	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.114	0.984	0.450	0.255	0.003	0.634	0.183	0.137	0.033	0.759	0.237	0.206
Butyric acid	0.067	0.218	0.126	0.054	0.023	0.197	0.113	0.074	0.051	2.598	0.768	1.067
Citric acid	0.366	0.516	0.442	0.060	0.019	0.882	0.177	0.260	0.008	2.305	0.450	0.670
Formic acid	0.043	0.193	0.118	0.087	0.008	0.648	0.176	0.221	0.037	0.845	0.252	0.200
Hippuric acid	-	-	-	-	0.010	0.029	0.019	0.010	0.006	0.117	0.025	0.027
Iso-butyric acid	-	-	-	-	-	-	-	-	0.077	1.880	0.719	0.461
Iso-valeric acid	0.039	0.151	0.080	0.055	0.015	0.224	0.079	0.066	0.085	1.105	0.376	0.322
Lactic acid	12.795	27.832	20.553	4.947	6.046	13.656	9.794	1.998	0.378	15.995	3.221	3.452
n-Valeric acid	0.603	3.501	1.657	1.433	0.210	2.332	1.272	0.595	0.269	0.685	0.518	0.220
Orotic acid	0.004	0.043	0.025	0.015	0.003	0.200	0.028	0.036	0.001	0.042	0.008	0.009
Oxalic acid	0.076	0.343	0.182	0.094	0.023	0.519	0.152	0.113	0.005	0.143	0.043	0.035
Propionic acid	0.021	0.074	0.045	0.019	0.002	0.091	0.045	0.022	0.110	0.489	0.232	0.092
Pyruvic acid	0.009	0.109	0.050	0.033	0.006	0.191	0.060	0.044	0.014	2.288	0.474	0.716
Uric acid	0.000	0.002	0.001	0.001	0.000	0.002	0.001	0.001	0.451	12.395	2.751	2.515
Lactose	2.430	13.285	7.744	3.539	0.655	22.855	11.216	4.838	0.145	73.975	10.250	15.487
Galactose	0.114	4.070	0.644	1.251	0.006	0.848	0.256	0.182	0.015	0.471	0.103	0.113
Glucose	-	-	-	-	0.010	0.400	0.096	0.098	0.174	4.370	1.336	1.112
Acetoin	0.001	0.025	0.007	0.008	0.000	0.013	0.005	0.004	-	-	-	-
Diacetyl	0.394	0.731	0.598	0.124	0.196	0.348	0.275	0.067	-	-	-	-

Table 3 - (continued).

	TAL				TLB				TMELL			
	m	M	Mean	SD	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.035	0.314	0.109	0.137	0.005	0.620	0.228	0.199	0.215	0.235	0.225	0.010
Butyric acid	-	-	-	-	0.005	0.895	0.252	0.291	-	-	-	-
Citric acid	0.545	1.615	1.230	0.458	0.010	0.840	0.141	0.244	0.005	1.255	0.427	0.717
Formic acid	-	-	-	-	-	-	-	-	-	-	-	-
Hippuric acid	0.005	0.040	0.023	0.025	0.005	0.050	0.016	0.014	0.003	0.008	0.005	0.003
Iso-butyric acid	0.847	0.863	0.855	0.008	0.002	0.009	0.005	0.004	0.003	0.008	0.005	0.003
Iso-valeric acid	0.005	0.375	0.190	0.262	0.015	1.215	0.469	0.365	0.014	0.026	0.020	0.006
Lactic acid	1.195	15.155	7.527	5.006	0.075	23.865	11.621	6.556	11.470	14.355	12.943	1.443
n-Valeric acid	0.005	0.340	0.100	0.161	0.005	0.570	0.073	0.187	0.002	0.008	0.005	0.003
Orotic acid	0.010	0.110	0.038	0.038	0.005	0.075	0.024	0.019	0.010	0.050	0.030	0.028
Oxalic acid	0.260	0.450	0.312	0.070	0.005	0.205	0.053	0.067	0.030	0.170	0.095	0.071
Propionic acid	0.012	0.033	0.022	0.011	0.003	0.400	0.171	0.195	0.002	0.004	0.003	0.001
Pyruvic acid	0.010	0.100	0.037	0.036	0.005	0.905	0.118	0.207	0.055	0.175	0.107	0.062
Uric acid	0.001	0.003	0.001	0.001	0.001	0.012	0.004	0.003	0.001	0.002	0.001	0.001
Lactose	6.080	23.810	19.488	6.766	0.005	1.080	0.287	0.445	0.475	2.730	1.457	1.155
Galactose	0.030	9.447	1.920	3.696	0.004	0.280	0.058	0.086	0.099	9.536	3.334	5.372
Glucose	0.005	0.615	0.293	0.306	0.005	0.135	0.060	0.038	0.092	0.098	0.095	0.003
Acetoin	0.002	0.027	0.009	0.009	0.001	0.035	0.006	0.009	0.001	0.005	0.003	0.002
Diacetyl	0.010	0.330	0.136	0.132	0.065	4.020	1.176	0.946	0.540	0.740	0.640	0.141

	TP				TRA			
	m	M	Mean	SD	m	M	Mean	SD
Acetic acid	0.023	3.290	0.557	0.531	0.023	0.047	0.035	0.012
Butyric acid	0.010	1.380	0.374	0.310	-	-	-	-
Citric acid	0.001	4.035	0.876	1.122	0.755	1.430	1.178	0.296
Formic acid	0.016	0.916	0.227	0.189	-	-	-	-
Hippuric acid	0.001	0.014	0.007	0.004	0.005	0.090	0.047	0.043
Iso-butyric acid	0.001	0.580	0.038	0.089	0.005	0.855	0.430	0.601
Iso-valeric acid	0.001	1.102	0.140	0.167	0.365	0.385	0.375	0.010
Lactic acid	1.986	35.530	16.629	8.496	2.755	7.035	4.600	2.200
n-Valeric acid	0.006	5.382	0.973	1.145	0.002	0.008	0.005	0.003
Orotic acid	0.001	0.302	0.020	0.026	0.010	0.065	0.044	0.025
Oxalic acid	0.006	0.760	0.178	0.185	0.040	0.450	0.250	0.169
Propionic acid	0.015	7.098	0.455	0.753	0.099	0.107	0.103	0.004
Pyruvic acid	0.003	0.595	0.064	0.073	0.005	0.060	0.023	0.032
Uric acid	0.000	0.110	0.010	0.015	0.001	0.002	0.001	0.001
Lactose	0.005	11.125	3.018	3.198	24.370	30.370	27.035	2.488
Galactose	0.000	7.782	1.419	1.943	0.025	0.368	0.225	0.146
Glucose	0.010	4.065	0.470	0.785	-	-	-	-
Acetoin	0.001	0.105	0.015	0.019	0.001	0.033	0.010	0.015
Diacetyl	0.003	4.198	0.460	0.772	0.010	0.130	0.083	0.064

Table 4 - Results of variance analysis and Duncan's test performed on all parameters determined by HPLC for all cheese varieties. Mean values (mg/g of cheese) in the same column followed by the same letter are not significantly different (p≥0.05). See Table 1 for cheese codes.

Cheese	Acetic acid	Butyric acid	Citric acid	Formic acid	Hippuric acid	Iso-butyric acid	Iso-valeric acid	Lactic acid	n-Valeric acid	Orotic acid
BD	0.835 cd	0.215 a	0.544 a	-	-	-	0.303 abc	26.311 i	1.862 c	0.012
BT	0.361 abc	0.142 a	1.419 ab	-	0.003 a	-	0.205 abc	17.934 efgh	1.293 ab	0.021
CA	0.737 bcd	0.377 a	0.114 a	0.065 a	-	-	0.472 c	14.832 cdef	1.971 c	0.012
CE	0.283 abc	0.110 a	0.023 a	-	-	0.095 ab	0.230 abc	11.743 bcde	0.005 a	0.015
MA	0.390 abc	0.258 a	1.370 a	0.038 a	0.010 a	-	0.243 abc	19.965 fgh	1.836 c	0.011
MU	0.352 abc	0.115 a	1.305 a	0.089 a	-	-	0.429 bc	24.618 hi	0.869 abc	0.016
MUR	0.155 a	0.274 a	0.047 a	-	-	-	0.794 d	9.278 abcd	0.005 a	0.017
PL	1.093 d	0.513 ab	0.444 a	0.681 b	-	0.048 a	0.235 abc	17.381 efgh	0.855 abc	0.024
RA	0.583 abcd	0.177 a	1.322 a	0.369 ab	-	-	0.236 abc	22.773 ghi	1.252 abc	0.031
RdA	0.450 abc	0.126 a	0.442 a	0.118 a	-	-	0.080 ab	20.553 ghi	1.657 b	0.025
RR	0.183 ab	0.113 a	0.177 a	0.176 a	0.019 ab	-	0.079 ab	9.794 abcd	1.272 abc	0.028
SA	0.237 ab	0.768 b	0.450 a	0.252 ab	0.025 ab	0.719 cd	0.376 bc	3.221 a	0.518 abc	0.008
TAL	0.109 a	-	1.230 a	-	0.023 ab	0.855 d	0.190 abc	7.527 abc	0.100 ab	0.038
TLB	0.228 ab	0.252 a	0.141 a	-	0.016 a	0.005 a	0.469 c	11.621 bcde	0.073 ab	0.024
TMELL	0.225 ab	-	0.427 a	-	0.005 a	0.005 a	0.020 a	12.943 cdef	0.005 a	0.030
TP	0.557 abc	0.374 a	0.876 a	0.227 a	0.007 a	0.038 a	0.140 abc	16.629 abcd	0.973 abc	0.020
TRA	0.035 a	-	1.178 a	-	0.047 b	0.430 b	0.375 bc	4.600 ab	0.005 a	0.044
Significance	***	***	***	***	**	***	***	***	***	ns

(*** p≤0.001; ** p≤0.01; * p≤0.05; ns not significant).

Cheese	Oxalic acid	Propionic acid	Pyruvic acid	Uric acid	Lactose	Galactose	Glucose	Acetoin	Diacetyl
BD	0.322 d	1.757 b	0.117 a	0.001 a	-	0.025 a	-	0.006 a	0.512 ab
BT	0.129 abc	0.435 a	0.025 a	0.003 a	0.013 a	2.894 bc	-	0.009 a	0.765 ab
CA	0.140 abcd	0.433 a	0.093 a	0.001 a	0.015 a	0.037 a	-	0.007 a	0.536 ab
CE	0.064 ab	0.424 a	0.024 a	0.001 a	1.113 a	0.227 a	0.053 a	0.002 a	1.178 bc
MA	0.163 abcd	0.308 a	0.035 a	0.001 a	-	2.423 abc	-	0.003 a	1.124 bc
MU	0.307 cd	0.380 a	0.157 a	0.002 a	1.409 a	0.769 ab	-	0.013 a	0.409 ab
MUR	0.160 abcd	0.472 a	0.171 a	0.026 a	0.010 a	0.061 a	0.053 a	0.006 a	1.898 c
PL	0.092 ab	1.411 b	0.159 a	0.012 a	1.581 a	0.330 a	0.333 a	0.058 b	0.341 ab
RA	0.153 abcd	0.473 a	0.101 a	0.002 a	0.135 a	1.323 abc	0.020 a	0.009 a	0.677 ab
RdA	0.182 abcd	0.045 a	0.050 a	0.001 a	7.744 ab	0.644 ab	-	0.007 a	0.598 ab
RR	0.152 abcd	0.045 a	0.060 a	0.001 a	11.216 b	0.256 a	0.096 a	0.005 a	0.275 ab
SA	0.043 a	0.232 a	0.474 b	2.751 b	10.250 ab	0.103 a	1.336 b	-	-
TAL	0.312 cd	0.022 a	0.037 a	0.001 a	19.488 bc	1.920 abc	0.293 a	0.009 a	0.136 a
TLB	0.053 a	0.171 a	0.118 a	0.004 a	0.287 a	0.058 a	0.060 a	0.006 a	1.176 bc
TMELL	0.095 ab	0.003 a	0.107 a	0.001 a	1.457 a	3.334 c	0.095 a	0.003 a	0.640 ab
TP	0.178 abcd	0.455 a	0.064 a	0.010 a	3.018 a	1.419 abc	0.470 ab	0.015 a	0.460 ab
TRA	0.250 bcd	0.103 a	0.023 a	0.001 a	27.035 c	0.225 a	-	0.010 a	0.083 a
Significance	***	***	***	***	***	***	***	***	***

(*** p≤0.01; ** p≤0.05; ns not significant).

the disappearance of galactose has been demonstrated (MICHEL and MARTLEY, 2001). The cheeses studied are generally produced with raw milk so differences in the galactose content could be explained by the different indigenous microflora present in each product.

Diacetyl and acetoin are produced from lactose and citric acid and are very important compounds for cheese aroma. The diacetyl and acetoin concentrations varied from 0 to 4.3 and from 0 to 0.2 mg/g of cheese, respectively. These values were not only directly correlated with ripening time but also with bacterial activity since higher values were recorded in cheeses like Toma del Lait brusc or Robiola di Roccaverano PDO which are produced with sour milk or in cheeses generally produced with a starter addition such as Bra tenero PDO or Bra duro PDO.

Among the organic acids, lactic acid was the most abundant ranging from 0.3 to 39 mg/g of cheese. It is produced by carbohydrate hydrolysis and its concentration is correlated with ripening time (PARK and DRAKE, 2005; PARK *et al.,* 2006); it increases in the initial phases and then diminishes during ripening (AKALIN *et al.,* 2002; FACCIA *et al.,* 2004). This reduction is probably due to the activity of lactate-fermenting bacteria as suggested by trends in the formic, propionic and butyric acids that are higher in long-ripening cheeses.

Citric acid is also quantitatively very important with a maximum of 4 mg/g in the Toma Piemontese PDO cheese. This acid is involved in the Krebs cycle where it acts as both substrate and product and its concentration is higher for cheeses with a short ripening time. Citrate can also be used by starter as a substrate to produce pyruvic and acetic acids. The concentrations of these compounds ranged from 0 to 2.2 mg/g of cheese and from 0 to 3.3 mg/g of cheese, respectively, and were higher in the longer ripened cheeses.

The presence of carboxylic acids like formic, acetic, propionic, butyric, valeric and iso-valeric acids is mainly related to the intensity of bacterial fermentation that takes place during ripening. These compounds are formed by the lipolytic activities of starters and secondary microflora of cheese. The highest concentration of formic acid (2.9 mg/g of cheese) was found in a Plaisentif sample, but this cheese also showed the highest mean values. This can be explained by the long ripening time of this cheese and by the fact that raw non-skimmed cow milk was used by all of the producers. The highest value for acetic acid (3.3 mg/g of cheese) was recorded in a Toma Piemontese PDO sample, while the highest values for propionic, butyric, iso-butyric, valeric and iso-valeric acids were 7.1, 2.6, 1.9, 5.4, 2.3 mg/g of cheese, respectively.

The maximum uric acid concentration (12 mg/g of cheese) was detected for Saras del Fen, a ripened ricotta cheese. Lower concentrations ranging from 0.002 to 0.14 mg/g of cheese were reported previously (LUES *et al.*, 1998; AKALIN *et al.*, 2002; TORMO and IZCO, 2004; PARK and DRAKE, 2005; MANOLAKI *et al.,* 2006; PARK and LEE, 2006), 0.67 m/g of milk (TORMO and IZCO, 2004) or 0.005 mg/mL of whey (KINIK and GÜRSOY, 2004). Further studies may be needed to clarify the differences between the reported data and the uric acid concentration determined in Saras del Fen.

Orotic acid concentrations varied from 0 to 0.3 mg/g of cheese with a mean value of 0.019 mg/g of cheese; the concentration of this acid in the milk depends on the origin, diet and lactation of the cow (ANASTASI *et al.,* 2000), while in the cheese, its relationship with ripening has not been defined. For some authors, the concentrations are lower in ripened cheeses because orotic acid is readily used by various bacteria (BOUZAS *et al.*, 1991; PARK and DRAKE, 2005;

MANOLAKI *et al.*, 2006) but for other authors the orotic acid concentration is higher in ripened cheese (AKALIN *et al.*, 2002). Still other authors have reported that the orotic acid concentration is independent of ripening time (LUES *et al.*, 1998; CALIFANO and BEVILACQUA, 1999; PARK and DRAKE, 2005). In the present study, the orotic acid concentrations were similar among all the Piedmont cheeses, independent of the ripening time.

Oxalic acid concentrations varied from 0 to 0.76 mg/g of cheese with a mean value of 0.099 mg/g of cheese. These values are similar to those reported in the literature; no differences due to ripening time were noted (LUES *et al.* 1998; TORMO and IZCO, 2004).

CONCLUSIONS

The present study is the first to determine the organic acid, sugar and ketone contents in typical Piedmont cheeses. These compounds are very important for the flavour profiles of the cheeses. Variability was very high for all the examined cheeses due to the non-standardised home-made production methods. Nevertheless, statistical differences can be highlighted for all the parameters examined. All of the cheeses investigated can be defined by a unique chemical profile that can be used to support an eventual request for a PDO designation. According to the published data the sugar concentration is inversely correlated to ripening time. The reported concentrations of acids and ketones are probably due to differences in the cheese-making procedures used by the various producers to make the same cheese. Further studies will be carried out on each of these typical cheeses in order to better define the interactions between the cheese-making parameters and the concentration of these compounds in the final product.

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Revised paper received May 1, 2007 Accepted June 25, 2007

IDF UNVEILS REVAMPED WEBSITE

Brussels, 9 January 2008 - The IDF has unveiled its revamped website. In addition to a new look and feel, and improved content, the redesigned website provides direct access to an additional brand new IDF site focusing on nutritional aspects of milk and milk products at http://www.idfdairynutrition.org

The revamping of the site reaffirms the commitment of IDF to help the online visitor find relevant information with more user friendly features. In addition, there are increased links between the various sections of the site so that in a specific area of work, the visitor can see the publications, relevant information and events matching this area. Print-friendly versions of the web pages are also available. All of these improvements are designed to find useful information faster and easier than before.

NEW IDF BOARD FAST TRACKS SUSTAINABILITY INITIATIVE

Brussels, 30 January 2008 - Sustainability and the environment are to be the focus of heightened activity at the International Dairy Federation.

IDF's new directors approved an ambitious programme aimed at improving the global dairy industry's environmental positioning. A work schedule is being drawn up by IDF's Science and Programme Coordination Committee (SPCC) and additional resources have been earmarked by the board to give the initiative early impetus. IDF President, Jim Begg said: We are acutely conscious of the potential impact on the dairy industry around the world of issues such as climate change and carbon footprinting. There is a global role for providing good balanced information in this area, particularly on the environment, so that developments are influenced by sound science. We aim to secure a leading role for the IDF in this area. We also intend to develop best practice in the key environmental areas relevant to the dairy industry. We shall do this using our own resources and by working in partnership with other organisations active in this area, such as the Sustainable Agriculture Initiative Platform. IDF Director-General, Christian Robert, said: IDF is already leading projects on environmental issues such as fresh water treatment, re-use of processed water, cutting on-farm emissions of greenhouse gases, effluent treatment and reduction of dust emissions from dairy plants.

For further information, please contact: Marylene Tucci, IDF Communications Officer - Tel. +32 2 7068644; Fax +32 2 7330413; e-mail: MTucci@fil-idf.org

6TH INTERNATIONAL CONFERENCE ON NUTRITION AND FITNESS
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The goals and objectives of the conference are to:
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of the various dietary sources of energy on energy expenditure, exercise and performance.
– Determine the relationship of nutrition and fitness to chronic diseases, particularly, the metabolic changes that occur with the type and amount of physical activity for the prevention and management of cardiovascular disease, cancer, obesity, Syndrome X, diabetes and osteoporosis.
– Consider the psycho-social and other determinants of physical activity throughout the life cycle including intervention strategies, and emphasize healthy lifestyles consistent with proper nutrition and fitness.
– Stimulate national governments and the private sector to coordinate and thus maximize their efforts to develop programs that encourage proper nutrition and participation in sports activities by all, throughout the life cycle, to achieve their potential in fitness and thus increase the pool of young athletes, from whom the elite athlete will be forthcoming.
– Develop strategies for the distribution and implementation worldwide of the 1996 "Declaration of Olympia on Nutrition and Fitness" for the New Millennium.

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Deadline for abstracts to be considered for oral or poster presentation is February 29, 2008. Notification of acceptance is March 31, 2008. Please send a one-page single-spaced abstract with a copy to Mrs. Maglara-Katsilambrou (estasinou@tmg.gr) and a copy to Dr. Simopoulos (cgnh@bellatlantic.net).Web: http://6nutritionfitness.tmg.gr/

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GUIDE FOR AUTHORS

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CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2008.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2008.

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ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 6.00

ISSN 1120-1770 © 2008

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

















VOLUME XX IJFS No. 1, 2008

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